Analysis of sex chromosome effects on behaviour

This thesis is submitted for the degree of Doctor of Philosophy
at Cardiff University

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Summary

Sexual dimorphisms are seen in healthy individuals and in disorders. Recent research in model systems has challenged the dogma that sexual dimorphisms in the brain arise solely from sex differences in gonadal hormones; such studies have suggested that products encoded by sex-linked genes may act directly on the brain, interacting with or independently of gonadal hormones, to contribute to aspects of neural sexual dimorphism.

In this thesis, two mouse models were used to determine the extent to which gonadal hormones and sex-linked genetic mechanisms underpinned aspects of behaviour.

The first of these models, the XO mouse, enables us to identify brain processes influenced by X-monosomy (i.e. lack of an X chromosome), or X-linked genomic imprinting (i.e. the parental origin of the single X chromosome). The second of these models, the Four Core Genotypes (FCG) cross allows a dissociation between brain and behavioural effects due to the action of the Y-linked gene *Sry* (either direct brain effects, or indirect effects on gonadal hormone secretion) and of other sex-linked genes.

Data from the XO mouse model suggested an X-monosomy effect on the acquisition of two biconditional discriminations, but no effects on response conflict, as measured by a novel murine version of the 'Stroop task'; the X-monosomy effect was not due to non-specific effects on physiology or behaviour. Data from the second model suggested *Sry*-dependent effects on anxiety (as indexed by the elevated zero maze) and on initial acquisition of a stimulus-reinforcer contingency in a two-way visual discrimination paradigm, but not on reversal learning. Assays of systemic testosterone levels and *Sry* brain expression in FCG mice and wildtype males indicated that the anxiety phenotype may be primarily due to testosterone, whilst the acquisition effect was more likely to be due to *Sry* expression in the brain.

These data highlight the importance of direct effects of sex-linked genes on brain and behaviour. They may be of relevance to understanding the mechanisms underlying neuropsychological abnormalities in disorders, such as Turner syndrome (X-monosomy) and XYY, and sexual differentiation of the brain in mammals.

List of abbreviations

5-HT	serotonin
5-HIAA	5-hydroxyindoleacetic acid (a metabolite of serotonin)
AD	Alzheimer's disease
ADHD	attention-deficit hyperactivity disorder
ANOVA	analysis of variance
AR	androgen receptor
BD	bipolar disorder
BOLD	blood oxygen level-dependent
CC	corpus callosum
cDNA	complementary DNA
CRF	continuous reinforcement
CS	conditional stimulus
CT	computed tomography
C_T	crossing threshold
DA	dopamine
DBD	DNA-binding domain
df	degrees of freedom
DHT	dihydrotestosterone
DNA	deoxyribonucleic acid
D-KEFS	Delis-Kaplan Executive Function System
DOPAC	3,4-dihydroxyphenylacetic acid (a metabolite of dopamine)
dpc	days *post coitum*
DTI	diffusion tensor imaging
E	estradiol
EEG	electroencephalography
ELISA	enzyme-linked immunosorbent assay
ER	oestrogen receptor
ERE	oestrogen response element
FCG	Four Core Genotypes
FDi	fibre density index
Fdopa	[^{18}F]fluorodopa
fMRI	functional magnetic resonance imaging
GABA	γ-Aminobutyric acid
GDX	gonadectomised
HMG	high mobility group
Hprt	hypoxanthine guanine phosphoribosyl transferase
KS	Klinefelter's syndrome
IRI	inter-reinforcement interval
ITI	inter-trial interval
MAOA	monoamine oxidase A
LBD	ligand-binding domain
MRI	magnetic resonance imaging
MRS	magnetic resonance spectography
MS	multiple sclerosis
MWF	myelin water fraction
NACWO	Named Animal Care & Welfare Officer
NIMR	MRC National Institute of Medical Research
NP	nose poke
NRX	non-recombining region of the X
NRY	non-recombining region of the X

PAC	primary auditory cortex
Paf	'*patchy fur*' mutation
PAR	pseudoautosomal region
PCR	polymerase chain reaction
PD	Parkinson's disease
PET	positron emission tomography
PFC	prefrontal cortex
POE	Parent-of-origin effect(s)
RI	random interval
RNA	ribonucleic acid
qPCR	quantitative (real-time) polymerase chain reaction
SCR	skin conductance reactivity
SDN	sexually dimorphic nucleus
SEM	standard error of the mean
SPANOVA	mixed design ANOVA
SPECT	single photon emission computed tomography
SSRIs	selective serotonin reuptake inhibitors
Sry	sex-determining region Y
STG	superior temporal gyrus
TH	tyrosine hydroxylase
TS	Turner syndrome
TP	testosterone propionate
XCI	X chromosome inactivation

Chapter I

General Introduction

1.1 Sex differences in behaviour

There is a large body of scientific literature which shows that males and females of many diverse species (including mammals) behave differently. In mammals, these differences may manifest in terms of sexual behaviour (Pardridge *et al.*, 1982; Swaab, 2007), early postnatal behaviours (for example, suckling, sibling rivalry, mother-offspring interactions; Lonstein & De Vries, 2000; Lee & Moss, 1986), locomotion (Cho *et al.*, 2004), emotion (for example, aggression, anxiety, motivation; Kring & Gordon, 1998; Cahill *et al.*, 2004), feeding and drinking behaviours (Asarian & Geary, 2006; Butera, 2010), and cognition (Halpern, 1997; Kimura, 2002; Overman, 2004; Sommer *et al.*, 2004; Koscik *et al.*, 2009; Rushton & Ankney, 2009). In this first section, I examine evidence for sex differences in healthy individuals (focussing on data related to cognitive and emotional phenotypes in humans and rodents) and how sex may influence susceptibility to, and the course of, certain neuropsychiatric and neurodegenerative disorders. In this thesis, I consider 'sex differences', specifically referring to the variations in biology between men and women, rather than 'gender differences', which refer to the differences in both biology and self-representation as male or female as shaped by the environment (Cosgrove *et al.*, 2007).

1.1.1 Healthy individuals

Extensive data from humans and rodent models provide evidence for behavioural differences between the sexes across a range of emotional and cognitive domains. A small selection of these is summarised in **Table 1.1.1i**. Briefly, there are developmental differences in language between boys and girls; girls tend to learn to speak earlier and gain a larger vocabulary than boys (Kimura, 2000; Wallentin, 2009), and adult females were shown to have better spelling and grammar (Berninger *et al.*, 2008). In humans, men have been shown to perform better than women in some spatial tasks, such as navigation (Woolley *et al.*, 2010) and 3D mental rotation, while women outperformed men in spatial memory (Silverman *et al.*, 2007); similarly, male rodents have been found to exhibit an advantage over females in some measures of spatial ability and working memory (Jonasson, 2005). With regard to emotion, women have been shown to be

more emotionally expressive and to score more highly on measures of anxiety than men (Kring & Gordon, 1998; McLean & Anderson, 2009); however, on various animal tests of anxiety, female rodents were found to be less anxious than males (Johnston & File, 1991). In both humans and rodents, females were more sensitive to pain than men (Wiesenfeld-Hallin, 2005).

Table 1.1.1i Data from human and rodent studies that provide evidence for sex differences in various aspects of behaviour.

Species	Behavioural measure	Psychology	Details of sex difference	Reference
Human	EEG study with verbal creativity task	Verbal ability	During original responses, women with higher verbal ability showed stronger increases in alpha power than women with lower ability; this pattern was shown to be reversed in men.	Fink & Neubauer, 2006
Human	3-D mental rotation and object location memory test	Spatial ability and memory	Men outperformed women in 3-D mental rotation while women were better at spatial, object location memory.	Silverman *et al.*, 2007
Human	Assessment of emotional response after watching films	Emotion	Women were more emotionally expressive than men in response to emotional films. Men displayed greater skin conductance reactivity (SCR) towards fear and anger films, whereas women showed greater SCR towards sad and disgust films.	Kring & Gordon, 1998
Human	Review of various behavioural measures	Fear and anxiety	Self report studies indicated great number and severity of fears in women than men. Women also scored more highly on other anxiety-related factors, such as trait anxiety, worry and rumination.	McLean & Anderson, 2009 (review)
Human	Purdue Pegboard test	Motor skills and dexterity	Women outperformed men, indicating higher degree of motor dexterity.	Hall & Kimura, 1995
Human & rodents	Review of various behavioural measures	Pain sensitivity	For humans, rats and mice, females were found to be more sensitive to noxious stimuli than males. Oestrous cycle affected pain sensitivity in female rodents. Endogenous opioid systems underlying pain sensitivity might differ between male and female rodents.	Wiesenfeld-Hallin, 2005
Rats	Performance on elevated plus maze	Anxiety	Females spent more time in the open arms than males, suggesting that females were less anxious than males.	Johnston & File, 1991

Rats	Performance on Morris water maze	Spatial ability and memory	Generally males and females did not differ from each other in the time spent to find submerged platform. However, spatial learning (but not spatial memory) was affected by oestrous cycle of females.	Healy *et al.*, 1999
Rats	Performance on Y maze	Reversal learning	Females outperformed males in reversal learning, but no difference in initial acquisition.	Guillamón *et al.*, 1986
Rats & mice	Performance on water maze and radial maze	Spatial ability and memory	In the water maze, male rats exhibited a large significant advantage, but with mice, females showed a small advantage instead. With the radial maze, both male rats and mice exhibited larger advantages in working memory than female animals, but in mice, the sex difference was smaller.	Jonasson, 2005

When interpreting data regarding sex differences between healthy individuals one must exercise caution, as in some cases data are controversial with studies showing contradictory results within and between species (Wallentin, 2009; Jonasson, 2005). This may be due to small true effect sizes (difficult to detect reliably), differences in experimental paradigms used and/or fluctuating hormonal status due to oestrus cycle which could affect female behaviour substantially (Lacreuse, 2006; Sherwin, 2003; Wiesenfeld-Hallin, 2005; Healy *et al.*, 1999).

1.1.2 Sex differences in disease states

In addition to the presence of sexual dimorphisms in behaviour between healthy individuals, sex differences are also apparent in a variety of neuropsychiatric and neurodegenerative disorders in humans (Holden, 2005), which may be manifest in terms of incidence, developmental course, underlying neurobiology, response to therapy and prognosis (**Table 1.1.2i**). For example, sex differences are observed in many aspects of schizophrenia; whilst no sex differences were observed in prevalence of the disorder (Saha *et al.*, 2005), male incidence[1] is generally higher than that of females (McGrath *et al.*, 2004). A comprehensive review by Leung and Chue (2000) has

[1] Prevalence measures the proportion of people who have the disorder at a particular point in time; it is calculated by dividing the number of people with the disorder at a particular time point by the total number of people examined. Incidence measures the rate of occurrence of new cases of the disorder; it is calculated by dividing the number of new cases in a specific time period, usually a year, by the size of the population under consideration who are initially healthy (Crichton, 2000).

outlined sex differences in symptomatology, cognitive dysfunction, neurobiological and neurochemical abnormalities, treatment response and course of illness. Briefly here, males have an earlier age of onset by around three to five years than females; additionally, early-onset schizophrenia (first psychotic episode before age of 21) is associated with more severe symptomatology than late-onset schizophrenia, which is more frequent and more severe in women than in men (Häfner *et al.*, 1998). Males with schizophrenia generally exhibit more negative symptoms (e.g. social withdrawal, blunted affect, alogia and motivation), while females suffer from more affective symptoms (e.g. dysphoria, depression, impulsivity and sexual delusions) and auditory hallucinations (Castle & Murray, 1991). With regard to cognitive dysfunction, males who have been ill for five years or longer displayed greater deficits on verbal tasks (suggestive of a left hemisphere dysfunction), Wisconsin Card Sorting Test, and tests of attention, verbal memory and executive function, than females. There is less evidence suggesting greater cognitive impairment in women; some studies have shown that cognitive deficits might affect social functioning of females more than males. Regarding neurobiological abnormalities, males have shown larger ventricular-brain ratios, suggesting increased ventricular volume, than females (Nopoulos *et al.*, 1997). The reduced size of the temporal lobes (especially that of the left temporal lobe) is more prominent in males than in females (Crow, 1990), and whilst the corpus callosum has consistently shown abnormalities, the pattern of results has not always been consistent. Females have better short and middle term outcomes than males, but this advantage reduces over time, in the long term; this might be due to the loss of protective effects of oestrogen in females (Castle & Murray, 1991; Leung & Chue, 2000).

As with data from healthy individuals, it is important to be aware of the potential caveats associated with reported sex differences in neuropsychiatric and neurodegenerative disorders. There are conflicting results in some of the sex differences examined, which might be due to a range of confounding factors such as small number of subjects and different methodologies; there is also a need to take into account sexual dimorphic brain development, which might further confound results (Mahone & Wodka, 2008). Another major problem is that of ascertainment bias, whereby one sex may be preferentially diagnosed; for example, the lower referral rates to clinics might be due to a neglect of the problems seen in girls with ADHD and/or the nature of difficulties in girls (e.g. more learning problems, rather than the more disruptive and apparent conduct problems seen more often in boys; Gaub & Carlson, 1997). A similar problem might be present in the determination of prevalence of autism, in that there might be a 'hidden horde' of autistic children who remain undiagnosed due to the lack of obviously debilitating symptoms (Blaxill, 2002).

Table 1.1.2i Sex differences in a variety of neuropsychiatric and neurodegenerative disorders.

Disorder	Measure (and psychology)	Details of sex difference	Reference
Schizophrenia	Incidence and prevalence	Median incidence rate is 15.2 per 100,000 and male incidence is generally higher than females (McGrath *et al.*, 2004). However, no sex differences in prevalence are found (Saha *et al.*, 2005).	McGrath *et al.*, 2004; Saha *et al.*, 2005
Schizophrenia	Age of onset, disease course, prognosis	Males have earlier age of onset than females. Early onset schizophrenia has more severe symptoms and less favourable prognosis. Late onset schizophrenia is more frequent in females than males, and oestrogen might offer protection to females.	Häfner *et al.*, 1998
Schizophrenia	Neurobiology	Male sufferers have significantly larger ventricles than male controls, but female sufferers did not differ significantly from female controls. Male and female patients have similar pattern of structural brain differences, but males have a greater number of significant abnormalities than females.	Nopoulos *et al.*, 1997
Schizophrenia	Symptoms, cognitive impairments, response to therapy	Males have more negative symptoms and cognitive impairments, while females show more affective symptoms and auditory hallucinations. Pre-menopausal females respond to antipsychotics more effectively but with more side effects.	Leung & Chue, 2000
Unipolar depression	Prevalence	Unipolar depression is more common in females than males by a factor of about 2.	Maier *et al.*, 1999
Unipolar depression	Neurobiology, response to therapy	Some evidence that hippocampal volume increases in females, but decreases in males. Female responders to antidepressant treatment have larger hippocampal volume than female non-responders, but no pattern seen in males. Females have smaller amygdalae but this is not observed in males. Some evidence for a reduction in medial orbitofrontal cortex in males only.	Lorenzetti *et al.*, 2009
Unipolar depression	Symptoms	Females report more vegetative symptoms (e.g. increased appetite), anxiety and anger than males. Self report of depressive symptoms is more severe in females than males. There were no sex differences in disease course and response to treatment.	Scheibe *et al.*, 2003
ADHD	Prevalence, cognitive impairment	ADHD occurs primarily in males, with prevalence varying from 9:1 to 6:1. Females	Gaub & Carlson, 1997

		have lower hyperactivity, fewer conduct disorder diagnoses, fewer externalising behaviours (e.g. aggression, hyperactivity), but greater intellectual impairment, than males. It is not known whether referral bias plays a part in these differences (see text).	
ADHD	Neurobiology	Some evidence that whilst males show a difference in right-left caudate asymmetry, females did not show this difference in asymmetry; instead there was a difference in left caudate volume and total caudate volume.	Castellanos *et al.*, 2001
ADHD	Neurobiology, cognitive impairments	Boys with ADHD exhibited a less right-lateralized frontal alpha asymmetry than control boys, whereas girls with ADHD displayed a more right-lateralized asymmetry pattern than control girls. Motor development and oculomotor control show sex differences between boys and girls with ADHD. Boys and girls with ADHD are best classified using different D-KEFS[2] tests; boys and girls are best classified with tests involving speed and efficiency of responding, and visuospatial planning, respectively.	Mahone & Wodka, 2008
Parkinson's disease (PD)	Prevalence, symptoms, response to treatment	Prevalence is higher in males than in females. Oestrogen appears to provide protective effect. Women with PD have reported greater disability and reduced quality of life than men, though reported quality of life correlates poorly with disease severity. Levodopa, the standard treatment for PD, is metabolised differently between males and females.	Shulman, 2007
Alzheimer's disease (AD)	Prevalence	There is evidence that prevalence is higher in females than in males (however incidence does not show a gender difference).	Compton *et al.*, 2002
Alzheimer's disease (AD)	AD pathology	A higher level of AD pathology is found in females. The association between AD pathology (neuritic plaques and neurofibrillary tangles) and clinical AD is stronger in females than in males. AD pathology is more likely to be expressed clinically as dementia in women.	Barnes *et al.*, 2005

[2] Delis–Kaplan Executive Function System (Trail Making, Verbal Fluency, Color–Word Interference, Tower) cognitive test

1.2 Mechanisms of sex differences

1.2.1 Brain differences

The proximal cause of sex differences in behaviour in healthy individuals (and in disease states) is sex differences in brain structure and function. Traditionally, sex differences in brain structure were determined *post mortem* through histological examination; more recently, brain structure may be investigated through *in vivo* imaging methods such as computed tomography (CT) and magnetic resonance imaging (MRI). CT creates 3-D images from many 2-D X-ray images, and MRI detects the rotating magnetic field in (usually) hydrogen atoms which has been aligned with a large magnetic field and altered by radio frequency; MRI images provide much better contrast than those from CT. Sex differences in brain function may be examined using positron emission tomography (PET), single photon emission computed tomography (SPECT), or functional MRI (fMRI); both PET and SPECT use radioactive tracers and detect gamma rays released during radioactive decay of compounds, whilst fMRI measures blood oxygen level dependence (BOLD), i.e. the changes in the oxygenation of haemoglobin in blood (Cosgrove *et al.*, 2007). Function may also be measured using electroencephalography (EEG), which records electrical activity on the scalp, produced by firing of neurons within the brain, as an index of activity. Additionally, neural tracts and connectivity may be examined using diffusion tensor imaging (DTI), which measures the restricted diffusion of water between brain regions.

1.2.1.1. Structural and functional differences

There is accumulating evidence, from both healthy humans and rodents (**Table 1.2.1.1i**) and humans with neuropsychiatric and neurodegenerative disorders (**Table 1.2.1.1ii**), for sex differences in structure and function of certain regions of the brain using the techniques described above. Briefly, whole female brains have been shown to be smaller in volume than male brains (Courchesne *et al.*, 2000), however, not all brain regions in females are proportionally smaller than males; there are specific brain regions which are bigger in females than in males (Brun *et al.*, 2009). Differences in brain activation in humans have been observed during a multitude of cognitive tasks, such as working memory (Goldstein *et al.*, 2005) and emotion recognition tests (Lee *et al.*, 2002; Hall *et al.*, 2004). Both structural and functional brain sex differences have been shown in a range of neurobiological disorders, ranging from panic disorders (neuropsychiatric; Asami *et al.*, 2009), to autism (neurodevelopmental; Bloss & Courchesne, 2007) and to multiple sclerosis (neurodegenerative; Antulov *et al.*, 2009, Pozzilli *et al.*, 2003). Additionally, brain symmetry has been changed differently between males and females in

bipolar disorder (Mackey *et al.*, 2010), and in humans with Alzheimer's disease, it would appear that males can compensate for neurodegeneration better than females (Perneczky *et al.*, 2007). These changes are likely to be of causal relevance to the behavioural differences reviewed above. Sex steroid receptors are likely to play an important role in establishing sex differences in the brain; Goldstein and colleagues (2001) have found greater sexual dimorphisms in particular human brain areas that coincide with analogous regions showing greater levels of sex steroid receptors in animal studies. Once again, however, data can be controversial in some cases; for example, whilst some studies have noted similar percentages of grey matter in both human females and males (Courchesne *et al.*, 2000), others have not replicated this finding and instead observed higher percentages in females than in males (Cosgrove *et al.*, 2007).

Table 1.2.1.1i Details on a selection of structural and functional brain sex differences in healthy humans and rodents.

Species	Technique	Brain region	Details of sex difference	Reference
Human	MRI	Intracranial space, whole brain	Intracranial space and whole brain, across the ages 19 months to 80 years, is about 10% and 12% smaller in females than in males, respectively.	Courchesne *et al.*, 2000
Human	MRI	Temporal, parietal and occipital lobes, anterior cingulate	The following brain regions are proportionally larger in females than in males: left temporal, left parietal and left occipital lobes, left and right superior temporal gyri, anterior cingulate. Males have proportionally larger left superior lateral fasciculus than females.	Brun *et al.*, 2009
Human	fMRI	Thalamus, occipital, temporal, frontal, lentiform regions	Activation when viewing happy faces differ between males and females; while bilateral frontal and left parietal activation is observed in both sexes, females show left thalamic, right occipital and right temporal activation which is not seen in males. During viewing of sad faces, males demonstrate bilateral frontal, right temporal and right lentiform activation, whereas the female subjects show left parietal, left lentiform and right occipital activation, without activation of frontal regions.	Lee *et al.*, 2002
Human	fMRI	Prefrontal, parietal, cingulate, insula regions	During an auditory working memory task (with the performance of a simple attention task as baseline), women activated right orbitofrontal cortex, right Broca's area and left dorsolateral	Goldstein *et al.*, 2005

			prefrontal cortex (PFC) more than males. Performance of males and females on these cognitive tasks do not differ from each other.	
Human	DTI and myelin water fraction (MWF)	Corpus callosum (CC)	Males exhibit higher MWF in the CC than females, suggestive of increased myelin content and fibre density. With DTI, there is increased FDi[3] in females compared to males in the genu of CC.	Liu *et al.*, 2010
Human	PET	Primary auditory cortex (PAC)	Activation of the PAC is examined while subjects are listening to music, noise, or no auditory stimuli. The PAC is more activated by music than noise in both males and females, but this increase in activation is much higher in males than in females. Comparing activation by noise with baseline, the female PAC is activated more than male PAC. Furthermore, males display a deactivation in the right PFC, which is not seen in females, suggestive of sex differences in auditory attention.	Ruytjens *et al.*, 2007
Human	PET	Frontal cortex, limbic system	While recognising emotion in faces, females show bilateral frontal activation and males show right frontal activation only. If auditory emotional stimuli are added, males show left frontal activation, and females no longer display frontal activation, instead, they showed greater limbic activation.	Hall *et al.*, 2004
Rat	*Post mortem*	Sexually dimorphic nucleus in the preoptic area (SDN)	The SDN is several folds larger in males than in females. Treating gonadectomised neonatal females with testosterone increased the SDN size, while gonadectomised males display decreased SDN size; however, the decreased size is still bigger than normal females, suggestive of genetic factors partially influencing SDN size.	Döhler *et al.*, 1982
Mouse	MRI	Whole brain, posterior hypothalamic area, CC, hippocampus, cerebellum, hypothalamus	Male whole brains are larger than female brains. Shape distortion between the sexes has been noted in the posterior hypothalamic area, CC, hippocampus, cerebellum and whole brain structure. For the following, the male region is larger than that of females: thalamus, primary motor cortex, posterior	Spring *et al.*, 2007

[3] Fibre density index, which is related to the total number of white matter tracts in the region

			hippocampus, and females are larger than males in posterior hypothalamic area, entorhinal cortex and anterior hippocampus.	
Mouse	MRI	Lateral ventricle, amygdala	Females have larger lateral ventricles and smaller amygdala than males. Lateral and third ventricles have sexually dimorphic developmental pattern with a reduction in sizes in males. Males display a lateralised enlargement in left amygdala which is not seen in females.	Koshibu *et al.*, 2004

Table 1.2.1.1ii Details on a selection of structural and functional brain sex differences in humans with neuropsychiatric and neurodegenerative disorders; additional sex differences in neurobiology of other disorders can be found in **Table 1.1.2i**.

Disorder	Technique	Brain region	Details of sex difference	Reference
Panic disorder	MRI	Amygdala, superior temporal gyrus (STG), insular cortex, lateral occipitotemporal gyrus, PFC, thalamus, parietal cortex, cerebellar vermis	Males show reduced right amygdala, bilateral insular cortex and left lateral occipitotemporal gyrus volumes compared to females. Females have greater right STG volume reduction than males. Females have reduced grey matter in bilateral dorsolateral and ventrolateral PFC, thalamus, parietal cortex and right cerebellar vermis than female controls, while male patients do not show this region-specific reduction.	Asami *et al.*, 2009
Bipolar disorder	MRI	Frontal, temporal, parietal, occipital lobes	Males with bipolar disorder tend to have larger left frontal, left temporal, right parietal and right occipital lobes when compared to control males, whereas females with the disorder tend to have smaller volumes in the above regions than healthy females. Brain asymmetry is also affected, with male patients displaying a more symmetric brain compared to control males, and female patients have more asymmetric brains than control females.	Mackey *et al.*, 2010
Autism	MRI	Temporal lobe, cerebellum	Females show enlargement in temporal white and gray matter volumes and reduction in cerebellar gray matter volume, when compared to males. Females, not males, display	Bloss & Courchesne, 2007

			an age-structure size relationship, in which age and white matter volumes are positively correlated.	
Parkinson's disease	SPECT	Caudate nucleus, putamen (striatum)	Females showed 16% higher striatal [^{123}I]FP-CIT binding than men at symptom onset and throughout the course of the disease, suggesting that critical threshold of dopamine depletion at which symptoms emerge is 16% higher in females than in males.	Haaxma *et al.*, 2007
Alzheimer's disease	PET	Inferior frontal, superior temporal and insular cortices, hippocampus	Using ^{18}F-FDG PET imaging, glucose metabolism in right inferior frontal, superior temporal and insular cortices, and hippocampus was reduced in males, when compared to females, at the same disease stage. This suggests that males can compensate for neurodegeneration better than females.	Perneczky *et al.*, 2007
Multiple sclerosis (MS)	MRI	Grey matter, white matter, lateral ventricles	Males showed less peripheral and total grey matter and more advanced central atrophy than females. White matter volume is lower in females than in males. This suggests males and females tend to have more severe grey and white matter atrophy respectively. In relapsing-remitting MS male patients, there is higher lateral ventricle volume.	Antulov *et al.*, 2009
Multiple sclerosis (MS)	MRI	Whole brain	Males have lower number of contrast-enhancing lesions but higher number of lesions evolving into black holes than females; this suggests that males are less prone to inflammatory lesions in the brain, but the lesions they do develop might be more destructive.	Pozzilli *et al.*, 2003

1.2.1.2 Neurochemical and molecular differences

Sex differences in brain structure and function in healthy subjects (and in humans with neuropsychiatric or neurodegenerative disorders) may be caused, or modulated by, sex differences in neurochemistry and gene expression. Neurochemistry may be measured *in vivo* by microdialysis or by receptor autoradiography (for example, a radioactive ligand specific to a neurotransmitter receptor might be injected and detected by PET or SPECT). The

neurochemistry of a particular patient group may also be inferred from their response to drugs targeting the brain, for example, specific serotonin reuptake inhibitors, SSRIs. **Table 1.2.1.2i** describes well established sex differences in neurochemistry in healthy humans and rodents; for example, serotonin (5-HT) displays sexual dimorphic activity in specific brain regions, in terms of concentration, synthesis rates, receptor binding, and transporter availability. Female rats have been shown to have higher 5-HT levels than males in a range of brain regions (ventromedial PFC, amygdala, insular cortex and dorsal hippocampus), whilst male rats displayed increased metabolite levels and turnover ratios in the same brain regions than females, which might signify general higher functional activity of 5-HT in males (Duchesne *et al.*, 2009); however, the result from this study might not reflect pure sex differences alone, as animals were subject to mild stress experience (not acute stress), and appears to be inconsistent with human imaging studies, which found healthy males to have a mean 56% higher synthesis rate than females through the brain (Nishizawa *et al.*, 1997). Women showed increased 5-HT_{1A} receptor binding potential in various brain regions, such as dorsal raphe, amygdala and anterior cingulate, than men (Parsey *et al.*, 2002); this might be due to sex steroids such as oestradiol, as it has been shown to modulate receptor binding potential (Biegon & McEwen, 1982). Brainstem 5-HT transporter availability was found to be significantly higher in women than in men (Staley *et al.*, 2001); again, this result might be due to sex steroids such as estradiol (McQueen *et al.*, 1997). The SDN has been consistently shown to be several folds larger in males than in females (Döhler *et al.*, 1982); male and female rats display sexual dimorphic distribution of serotonin-immunoreactive (ir) fibres in the SDN, with a larger region of low serotonin-stained fibre density in males compared to females (Simerly *et al.*, 1984).

Table 1.2.1.2i Details on a selection of sex differences in neurochemistry in healthy humans and rodents.

Species	Technique	Brain region	Details of sex difference	Reference
Human	[^{123}I]β -CIT SPECT and MRI	Striatum, diencephalic, brain stem	Females show significantly higher availability of striatal dopamine (DA) and diencephalic and brainstem serotonin (5-HT) transporters than males.	Staley *et al.*, 2001
Human	PET	Ventral striatum, anterior putamen, anterior and posterior caudate nuclei	Males display greater DA release in ventral striatum, anterior putamen, and anterior and posterior caudate nuclei than females.	Munro *et al.*, 2006

Human	PET	Striatum	Females displayed lower D_2 receptor affinity than males in the left striatum; this lower affinity suggests an increased endogenous striatal dopamine concentration in females.	Pohjalainen *et al.*, 1998
Human	PET	Dorsal raphe, amygdala, anterior cingulate, cingulate body, medial PFC, orbital PFC	Females display higher 5-HT_{1A} binding potential than males in all of the brain regions on the left.	Parsey *et al.*, 2002
Human	PET	Whole brain	The rate of synthesis of 5-HT is rather uniform throughout the brain. Males were found to have a 56% mean increase on the rate of 5-HT synthesis than females.	Nishizawa *et al.*, 1997
Human	PET	Striatum	Females have higher [^{18}F]fluorodopa uptake than males in the striatum; this difference is higher in the caudate than in the putamen. Higher [^{18}F]fluorodopa uptake shows higher striatal presynaptic DA synthesis capacity.	Laakso *et al.*, 2002
Human	Magnetic resonance spectroscopy (MRS)	Cortex	Females have higher cortical γ-aminobutyric acid (GABA) levels than males.	Sanacora *et al.*, 1999
Human	PET	Amygdala, thalamus, cerebellum	Females show higher mu-opioid receptor binding than males in amygdala, thalamus, and cerebellum (significant differences remain after accounting for multiple comparisons).	Zubieta *et al.*, 1999
Rats	Ligand binding and autoradiography	Cerebral cortex	Density of 5-HT_{2A} receptors in the cerebral cortex is higher in proestrous females than in males and diestrous females.	Fink *et al.*, 1998
Rats	*Post mortem*	Ventromedial PFC, amygdala, insular cortex,	Females and males show major and complex differences in levels of DA, DOPAC[4], 5-HT and 5-HIAA[5].	Duchesne *et al.*, 2009[6]

[4] DOPAC: Dopamine's major metabolite, 3,4-dihydroxyphenylacetic acid.

[5] 5-HIAA: Serotonin's major metabolite, 5-hydroxyindole acetic acid.

		dorsal hippocampus	Briefly, in the ventromedial PFC, females have higher levels of DA and 5-HT, and males have higher levels of DOPAC and 5-HIAA. In the amygdala, females have higher levels of 5-HT, while males show higher levels of DOPAC and 5-HIAA. In the insular cortex, females have higher levels of DA and 5-HT. Lastly, in the dorsal hippocampus, females have higher levels of 5-HT and 5-HIAA, with males having higher levels of DOPAC.	
Rats	*Post mortem*	Striatum	Male DA D_1 and D_2 receptor densities increase significantly more than that of females between 25 days of age and onset of puberty. The decline of receptor densities is also larger in males than in females.	Andersen & Teicher, 2000

There are sex differences in neurochemistry that have been reported in various neuropsychiatric and neurodegenerative disorders. Using SPECT, males with schizophrenia have shown a significant left side asymmetry in the concentration of striatal dopamine D_2 receptors, when compared with male controls; this pattern of results was not seen in females with schizophrenia (Acton *et al.*, 1997). In a PET study with bipolar disorder (BD) patients, males with BD were found to differ from male controls in 5-HT_{1A} binding affinity in a large range of brain regions, including anterior cingulate, amygdala, dorsolateral PFC, hippocampus, orbitofrontal cortex and temporal cortex, whilst females with BD did not differ from female controls in any of these regions (Sullivan *et al.*, 2009). Not all subjects for this study were antidepressant naïve and so this finding might reflect a sex-specific effect by antidepressants on 5-HT_{1A} binding. The large increase and decrease of the DA receptor density in striatum of male rats (Andersen & Teicher, 2000) might contribute to the sex differences in ADHD; the male-specific large increase of receptor density up to puberty mirrors the onset of motor symptoms in ADHD and the pruning of striatal dopamine receptors coincides with the 50-70% remission rate in ADHD, both at adulthood. Increased frontal monoaminergic activity has been observed in Parkinson's disease; using [^{18}F]fluorodopa(Fdopa)-PET, women with Parkinson's disease displayed 87% higher

[6] Note that the animals in this study had stressful experiences (e.g. via handling and restraint) during the experiment. The animals were sacrificed at least three hours after the last stressful experience, so the results reported here are likely to reflect intrinsic sex differences and repeated stress experience, rather than pure sexual dimorphisms.

Fdopa values in the right dorsolateral prefrontal cortex than men with the same condition, whilst there was no significant differences between male and female control subjects (Kaasinen *et al.*, 2001). As Parkinson's disease is characterised by a loss of dopaminergic activity in the striatum, this observed increase in frontal monoaminergic activity is rather paradoxical, and might reflect compensatory mechanisms; additionally, it is worth noting that Fdopa uptake is not specific to the dopamine system and might reflect an increase in serotonergic and noradrenergic activity instead. The sex difference in frontal monoaminergic activity might underlie other clinical sex differences, such as higher risk for comorbidity with depression in females with Parkinson's disease.

Gene expression is typically measured from *post mortem* tissue samples. The availability of microarray platforms has made it possible to assay the expression of many different genes in parallel, and thus to determine pathways which may give rise to sex-specific neuroanatomy and function in healthy (**Table 1.2.1.2ii**) and disease states. Differences in gene expression could exist in grey matter (Lopes *et al.*, 2006), whole brain (Yang *et al.*, 2006) or in specific brain regions (e.g. Vawter *et al.*, 2004, Zhang *et al.*, 1999). Some gene expression differences are determined by the sex chromosome complement rather than by gonadal hormones (see **Section 1.2.2** and **1.2.3** for more details; Xu *et al.*, 2008b), and it is important to remember that even though gene expression differences exist does not mean this difference will be apparent at the protein level (Xu *et al.*, 2006). If the difference disappears at the protein level, then arguably, there might be no differences between males and females functionally (though downstream effects at the gene level could still lead to differences in the levels of other affected proteins).

Table 1.2.1.2ii Details on a selection of sex differences in brain gene expression in healthy humans and rodents.

Species	Brain region	Details of sex difference	Reference
Human	Anterior cingulate, dorsolateral PFC, cerebellar cortex (*post mortem* samples)	Using Affymetrix oligonucleotide microarray and confirmed with *in situ* hybridisation, the genes *DBY*, *SMCY*, *UTY*, *RPS4Y*, and *USP9Y*, from the Y chromosome, are expressed more highly in the three brain regions in males than in females. The gene *XIST* on the X chromosome is expressed more highly in the three brain regions in females than males. *DBX*, *UTX*, *SMCX*, *RPS4X*, the X homologues, were not expressed differentially between males and females, suggesting that they do not escape X chromosome inactivation.	Vawter *et al.*, 2004

Human	Grey matter	*PCDH11X* expression is up to 2 fold higher in females than in males.	Lopes *et al.*, 2006
Rat	Hypothalamus, amygdala, hippocampus, ventromedial hypothalamic nuclei	5-HT_{1A} receptor mRNA expression is higher in hypothalamus and amygdala, and lower in hippocampus, in males compared to in females. 5-HT_{2A} receptor mRNA expression is lower in ventromedial hypothalamic nuclei in females compared to in males.	Zhang *et al.*, 1999
Rat	Amygdala, ventromedial hypothalamus	Males have reduced methyl-CpG-binding protein 2 (Mecp2) mRNA (and protein) than females in the amygdala and ventromedial hypothalamus on postnatal day 1. By postnatal day 10, this sex difference has disappeared and males have more mecp2 mRNA than females.	Kurian *et al.*, 2007
Mouse	Whole brain	Microarray study has found 612 genes that are expressed in a significantly sexual dimorphic manner in the brain. The authors examined gene expression in a range of tissue, including liver and adipose tissue, and found sexually dimorphic genes were expressed in a tissue specific manner.	Yang *et al.*, 2006
Mouse	Cortex, striatum, suprachiasmatic nucleus, paraventricular nucleus, hippocampus, dentate gyrus, habenula	The gene *Utx* from the X chromosome is expressed more highly in these brain regions in females than in males. *Utx* escapes X chromosome inactivation. The sex difference in expression appears to be dictated by sex chromosome complement rather than gonadal status, as XX animals display higher expression than XY animals regardless of gonadal status.	Xu *et al.*, 2008b
Mouse	Cortex, hippocampus, paraventricular nucleus	The X-linked gene *Eif2s3x* escapes X chromosome inactivation. *Eif2s3x* mRNA expression is higher in females than in males; however, this sexual dimorphic expression is restricted to mRNA only, at the Eif2s3x protein level, there is no difference between sexes, suggesting a possible regulation of the Eif2s3x protein translation independent of mRNA expression.	Xu *et al.*, 2006
Mouse	Preoptic area (POA)	Authors found sex-specific parent-of-origin allelic expression in the brain. Preferential expression of the paternal allele of the gene *Mrpl48* was found in the female POA, but not in the male POA. The gene *Il18* was found to be preferentially expressed from the maternal allele in the female, but not in the male mPFC.	Gregg *et al.*, 2010a

There are fewer studies and less evidence on sexual dimorphic gene expressions in the brain with regard to neuropsychiatric and neurodegenerative disorders. A recent study on Parkinson's disease has found different patterns of deregulation between men and women (Simunovic *et al.*, 2010); using post mortem brains and microarrays, downregulation of several genes from the PARK gene family was observed in males with PD only, and with pathway-enrichment and gene cluster analyses, it appeared that there was dysregulation of gene expression in PD-affected DA neurons in a sexually dimorphic manner, suggesting a male bias towards PD. Reduced expression of the gene Methyl-CpG-binding protein 2 (MeCP2) is implicated in autism, and in healthy rats, mecp2 mRNA expression appeared to be sexually dimorphic in amygdala, a region typically found to be aberrant in autism, during brain development, suggesting that lower MeCP2 expression in males might underlie the biased risk in autism and other neurodevelopmental disorders (Kurian *et al.*, 2007). *Il18*'s sex-specific parent-of-origin expression might be linked to multiple sclerosis, a sexual dimorphic neurodegenerative disease (Gregg *et al.*, 2010a).

1.2.2 The gonadal hormone dogma

1.2.2.1 Effects of gonadal hormones and their actions at receptors

The sexual differentiation of mammalian brain is largely determined by gonadal hormone secretions, i.e. from the male testes and the female ovaries. Hormonal effects in sexual dimorphism have been first demonstrated by Lillie (1917), who noted that in the cases of freemartins[7], the mixing of placental blood of male and female calves produced a more masculine and sterile female, leading to the suggestion that certain chemicals in the blood, such as 'sex hormones', can influence sex differentiation. Female guinea pigs were given testosterone prenatally and were found to display masculinised adult behaviour pattern (Phoenix *et al.*, 1959). Jost (1970) has suggested that the default developmental path for gonads, genital tract and brain is set for a female type, and masculinising agents and/or gonadal hormones would need to exert their effects at different phases in order to cause the developmental direction to deviate towards a masculine type.

The gonadal hormone that most obviously differentiates the two sexes is testosterone; in rats, it is actively produced in foetal Leydig cells within the testes by 15.5 dpc, with postnatal production

[7] "A condition occurring in the female offspring of dizygotic twins in a mixed sex pregnancy, usually in cattle. [...] When placental fusion between the male and the female foetuses permits the exchange of foetal cells and foetal hormones, testicular hormones from the male foetus can androgenise the female foetus producing a sterile XX/XY chimeric 'female'." (Quoted from National Library of Medicine)

continuing in adult Leydig cells that are distinct from foetal Leydig cells and arise from undifferentiated mesenchymal precursor cells. Lutenizing hormone is critical in the maturation of Leydig cells and maintenance of testosterone production (Wu *et al.*, 2007). In the brain, testosterone is either metabolized by aromatase to oestradiol or by 5α-reductase to dihydrotestosterone (DHT); oestradiol and DHT then act at oestrogen or androgen receptors respectively to contribute to sexual differentiation of the brain, in an organisational (i.e. during development) and activational (i.e. continuously during postnatal life) manner (Davies & Wilkinson, 2006). Note that normal fluctuations in oestrogen levels during the oestrus cycle in females can affect brain function and produce transient sex differences that may exist over and above persistent sexual dimorphisms. **Table 1.2.2.1i** outlines some of the sex differences that are influenced by changed in testosterone or oestrogen levels.

Table 1.2.2.1i shows that a variety of behaviours, including cognition, can be influenced by changes in gonadal hormone levels (testosterone or oestrogen). More studies can be found in **Table 6.1i**.

Hormone manipulations	Effect on neurobiology	Reference
Female humans; oestrogen replacement in menopausal women	Discrepancy exists between studies; it would appear that oestrogen has a protective effect on memory if it was administered immediately following surgical or natural menopause, but no effect if it was administered later.	Sherwin, 2006; Genazzani *et al.*, 2007
Male rhesus monkeys; testosterone administration	Supraphysiological testosterone levels in males impaired performance on the delayed nonmatching-to-sample test.	Lacreuse *et al.*, 2009
Male rats; gonadectomised (GDX) and gonadectomised with estradiol (GDX-E) or testosterone propionate (GDX-TP) replacement	GDX and GDX-E performed worse in response alternation and light/dark discrimination than control and GDX-TP animals. GDX animals showed impaired performance in the Differential Reinforcement of Low Rates of Responding task, compared the other groups. No effect of gonadectomy and hormone replacement on match-to-position and nonmatch-to-position tasks.	Kritzer *et al.*, 2007
Male rats; GDX, GDX-E, GDX-TP	GDX-E displayed enhanced acquisition of the delayed matching-to-position spatial task. GDX-TP performed better than GDX and GDX-E when the intertrial delay and working memory load was increased.	Gibbs, 2005
Male rats; GDX, with testosterone metabolite replacement	Androsterone is one of testosterone's metabolite and does not bind well with ERβ. 3α-diol decreased anxiety in the elevated plus maze and light/dark transition, and increased cognition in Morris water maze, while 3β-diol improved cognition in Morris water maze, but had no effects	Osborne *et al.*, 2009

	on anxiety behaviour, compared to control or androsterone. This suggests that actions at ERβ might underlie anti-anxiety and cognitive effects.	
Female rats; GDX and GDX-E	In the active avoidance task, GDX-E performed better than GDX animals after five weeks of hormone replacement. After 28 weeks, performance from GDX animals was impaired compared to control animals. GDX-E continued to perform better than GDX females and also displayed accelerated rate of learning. Choline acetyltransferase activity was decreased in the hippocampus of GDX animals, but not in GDX-E females, after five weeks. High-affinity choline uptake reduced in both frontal cortex and hippocampus in GDX animals, but no change was observed in GDX-E females.	Singh *et al.*, 1994

The two types of oestrogen receptors (ER), ERα and ERβ, are intracellular and present in the human and rat brain from early prenatal stages to adulthood in a distinctive spatial and temporal pattern (human: González *et al.*, 2007; rat: Pérez *et al.*, 2003; Kritzer, 2002, 2006), suggesting that the two receptor subtypes are involved in different and complimentary roles in brain development. ERβ is more highly expressed in the adult human hippocampus, neocortex and archicortex, supporting the suggestion that ERβ might be more involved in the function of the adult hippocampus, than ERα (González *et al.*, 2007). In addition to the hippocampus, both ERs are expressed in the frontal lobes, suggesting a role for oestrogen in higher cognitive functions (Sherwin, 2006; Genazzani *et al.*, 2007). ERs are ligand-activated protein transcription factor, which modulate specific gene expression by binding onto DNA sequences (Green, 1990); the ER comprises of six domains, with the regions A/B (the constitutive transcription factor 1 [TAF-1] binds onto this region), ligand-binding (LBD) and DNA-binding domains (DBD) being the three functional domains. An agonist can bind onto the LBD, resulting in dimerisation of the ER; the dimer then binds onto oestrogen response element (ERE) within a DNA sequence, via the DBD, activating transcription (Cano & Hermenegildo, 2000). ERα and ERβ have been shown to work in antagonistic, synergistic and sequential fashion when activating transcription (Cano & Hermenegildo, 2000) and when mediating aspects of cognition such as spatial learning (Rissman, 2008).

Androgen receptors (AR) primarily exist as intracellular nuclear receptors; while some immunoreactivity has been detected in the cytoplasm, indicating the presence of cytosolic receptors, antibody staining has displayed the highest immunoreactivity in the nucleus (Clancy *et*

al., 1992). Concentration of cytosolic AR tended to be higher in female than in male rats, while nuclear AR levels are higher in males than in females (Roselli *et al.*, 1989); it would appear that AR mRNA expression and protein level are dependent on androgen levels (Lu *et al.*, 1998; Lee & Chang, 2003). Nuclear AR were found in a wide range of rat brain regions, with the highest levels in the ventromedial nucleus of the hypothalamus and medial amygdala and lower levels in medial preoptic nucleus, periventricular preoptic area, anterior hypothalamus, periventricular anterior hypothalamus, lateral septum, and parietal cortex (Roselli *et al.*, 1989). This pattern of results largely matches that of AR mRNA expression, which also exhibited high levels in hypothalamus and in limbic regions that project heavily to the hypothalamus; these regions have been known to underlie copulatory behaviours (Simerly *et al.*, 1990). AR is a ligand dependent transcription factor, and upon binding onto androgens such as testosterone and 5α-dihydrotestosterone, the receptor undergoes two conformational changes, allowing the highly conserved DBD to bind onto androgen response elements (ARE) of target genes and activating transcription (Brinkmann *et al.*, 1999). Aside from the LBD and DBD, ARs are also composed of a variable NH_2-terminal domain, which contains polyglutamine (CAG) repeats whose length is inversely proportional to transcriptional activity *in vitro* (Lee & Chang, 2003). Longer CAG repeats in exon one of the AR gene has been associated with increased impairment of cognitive functioning in older men (Yaffe *et al.*, 2003), whilst 20 or fewer CAG repeats have been associated with increased AD risk in men but not in women (Raber, 2008). There is also increasing evidence that AR activity plays a role in hippocampal function (Raber, 2008).

1.2.2.2 Sex-determining region on the Y chromosome (Sry)

Seminal work in the 1990s showed that the Y-linked gene *SRY/Sry* (sex-determining region on the Y chromosome), encoding the testis-determining factor, is critical to gonad differentiation. In mice, the bipotential indifferent gonads are indistinguishable between the sexes until around 12 days *post coitum* (dpc; Wilhelm & Koopman, 2006), even though the differentiation of the gonads has already begun at 10.5 dpc, when SRY is transiently expressed for a few hours in the Sertoli cell precursors between 10.5 dpc and 12.5 dpc, with peak levels over the entire gonad at 11.5 dpc (Sekido & Lovell-Badge, 2009). In humans, SRY expression in normal males begins at around six to seven weeks in gestation (Davies & Wilkinson, 2006). *Sry* is a single exon gene (Clepet *et al.*, 1993) and encodes a high-mobility group (HMG) protein that acts as a transcription factor. The HMG box contains several functional motifs, such as nuclear localisation signals and calmodulin-binding site (Sekido & Lovell-Badge, 2008), and can bind to and bend DNA; aside from the HMG box, there is little structural conservation between species (Wilhelm &

Koopman, 2006). In mice, the SRY protein sets off the testis-determining pathway by directly binding, along with steroidogenic factor 1 (SF1), onto several elements within the *Sox9* enhancer to allow *Sox9* expression; afterwards, SOX9 and SF1 proteins bind to the same enhancer to maintain *Sox9* expression in a positive feedback manner, even after *Sry* expression has ceased (Sekido & Lovell-Badge, 2008).

In females, in the absence of Y chromosome and thus SRY, the ovarian-determining pathway will initiate in the bipotential gonads. *Sry* alone is sufficient to create a male phenotype, as demonstrated in a study by Koopman and colleagues (1990), in which mice with a female karyotype (XX) but an autosomal *Sry* transgene developed as phenotypic males. As the testes are primarily responsible for the biosynthesis and secretion of testosterone (see above), *Sry* may be considered as a factor which indirectly influences brain masculinisation via initiation of testes differentiation and subsequent testicular secretion of gonadal hormones.

In males, SRY initiates a complex series of molecular events which culminate in testes development and the abrogation of female reproductive organs (Wilhelm and Koopman, 2006), which is briefly summarised in **Figure 1.2.2.2a**.

1.2.3 Non-gonadal hormone mechanisms

Whilst gonadal hormones undoubtedly play a major role in generating sex differences in brain structure and function (see above), new evidence from avian and mammalian models has emerged to suggest that hormone factors are not the sole mediator.

Arnold (1997) showed that while masculine development pattern can be induced in female zebra finches (ZW) with oestrogen, it was not possible to induce female development patterns in male zebra finches (ZZ) with anti-oestrogens and oestrogen synthesis blockers. A similar study in gynandromorphic chicken, has recently demonstrated a similar phenomenon; for example, wattle growth has been shown to be sensitive to testosterone, but as shown in **Figure 1.2.3a**, the size of the wattle is determined by cellular composition of the tissue, with the left side being comprised of more ZZ-containing cells (Zhao *et al.*, 2010). An indication that a similar scenario may be relevant to mammals

Figure 1.2.3a A gynandromorphic chicken, with the left side showing a larger wattle.

(Taken from Zhao *et al.*, 2010, with permission)

has come from data in rodents. Neurons in mice that were harvested before the production onset of gonadal hormones have been shown to undergo sexual differentiation according to the sex of the animal (Carruth *et al.*, 2002) and Dewing and colleagues (2003) found over 50 genes which were expressed in a sexually dimorphic manner in the mouse brain before the onset of gonadal hormone production. Together these data suggest that both the genetic sex of the brain cells and the surrounding hormonal milieu could potentially specify their function.

Figure 1.2.2.2a

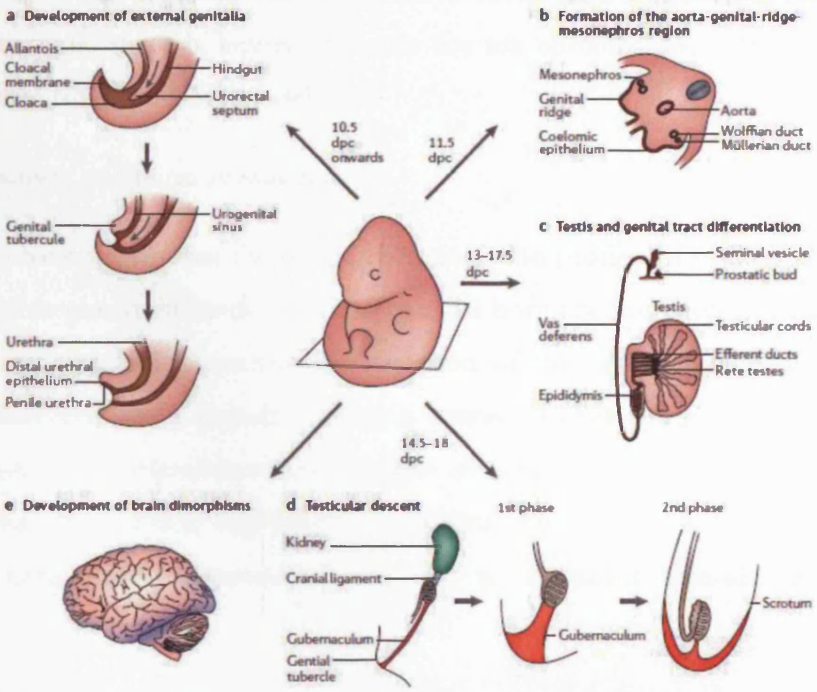


Figure 1.2.2.2a (a) At 10.5dpc, external genitalia appear as a swelling, (b) at 11.5dpc, males and females cannot be distinguished from each other morphologically, but in males, *Sry* expression induces a gene expression cascade, leading to differentiation of the genital ridge into testes, (c) and (d), steps of testes differentiation, (e) the brain develops sexual dimorphisms, caused by gonadal hormones and genetic factors. (Diagram taken from Wilhelm & Koopman, 2006, with permission)

Additional work in mice, using an experimental system known as the 'Four Core Genotypes' (FCG) model, in which the effects of sex chromosome complement (*Sry*-independent) and gonadal hormones (*Sry*-dependent) on brain and behavioural measures may be dissociated (see **section 1.2.4.2** for more details), has strengthened the notion that the mammalian brain too may be sensitive to both genetic sex and gonadal hormone effects. Briefly, this model generates XX animals with a *Sry* transgene (i.e. phenotypically male, but genetically female) and XY animals with *Sry* deleted from the Y chromosome (i.e. phenotypically female, but genetically male). Using this mouse model, to date, sex chromosome complement (i.e. *Sry*-independent) effects

have been described on measures as diverse as nociception (Gioiosa *et al.*, 2008), vasopressin immunoreactivity in lateral septum (De Vries *et al.*, 2002), habit formation (Quinn *et al.*, 2007), number of tyrosine hydroxylase (TH)-ir neurons in mesencephalon from embryonic day 14.5 (Carruth *et al.*, 2002), social interaction style (McPhie-Lalmansingh *et al.*, 2008), aggression and pup retrieval (Gatewood *et al.*, 2006), prodynorphin (*Pdyn*) expression in striatum (Chen *et al.*, 2009) and outcome devaluation (Barker *et al.*, 2010), while gonadal hormone (i.e. *Sry*-dependent) effects have been shown on various measures such as progesterone receptor immunoreactivity (Wagner *et al.*, 2004) and cortical thickness (Markham *et al.*, 2003). Both sex chromosome complement and gonadal hormone effects have been observed in immune response (Palaszynski *et al.*, 2005). To date, there is limited evidence for sex chromosome complement effects in cognitive measures from the FCG model.

1.2.3.1 Sex chromosome mechanisms in mammals

Data from mice have shown that the brain gene expression profiles of males and females differ at 10.5dpc, prior to gonad differentiation and gonadal hormone secretion (Dewing *et al.*, 2003). This suggests that the brain structure and function of the sexes may be different early in development, independent of gonadal hormone action. Unsurprisingly, several of the genes whose expression levels differed between the sexes are located on the sex chromosomes (i.e. the X or Y; Dewing *et al.*, 2003), highlighting a possible fundamental role for genes on these chromosomes (and their downstream effectors) in mediating sexually dimorphic brain phenotypes.

In mammals, males possess two sex chromosomes, an X (always inherited from their mother) and a Y (always inherited from their father). In contrast, females possess two X chromosomes (one inherited from their mother, and one from their father). The X chromosome is relatively large (~155Mb in humans, ~166Mb in mice; Ross *et al.*, 2005; Mueller *et al.*, 2008) and rich in genes associated with cognitive function (Nguyen & Disteche, 2006a, 2006b); in 2004, from the 1237 gene entries for 'mental retardation' in the Online-Mendelian Inheritance in Man, 27% mapped to the X chromosome (Zechner et al, 2001; Skuse *et al.*, 2005), whilst the Y chromosome is much smaller (~60Mb in humans, ~95Mb in mice; Skaletsky *et al.*, 2003; Bergstrom *et al.*, 1998) and is enriched for genes involved in spermatogenesis (Lahn & Page, 1997;). The two chromosomes recombine exclusively at sites towards their telomeres within pseudoautosomal regions (PARs; Ellis, 1998), where there exist X-Y homologous gene pairs (Heard & Dietsche, 2006); the remainder of the genes on the X and Y chromosome are located in the non-recombining region of the X (NRX) and non-recombining region of the Y (NRY)

respectively. Genes on the sex chromosomes may influence brain phenotypes via three possible genetic mechanisms:

(i) Y chromosome effects

Brain-expressed genes within the NRY lack functional homologues on the X chromosome and are unique to males (Xu *et al.*, 2002), hence have the capacity to influence male brain development and function specifically (Kopsida *et al.*, 2009). Of particular note here is *SRY*/*Sry*, the testis-determining gene referred to earlier in **section 1.2.2.2.** In addition to being expressed in the gonads during development and in adults, *Sry* is expressed in the mouse embryonic whole brain and postnatal midbrain, diencephalon and cortex (Mayer *et al.*, 2000), and in the substantia nigra of adult rats (Dewing *et al.*, 2006). In humans, *SRY* is expressed in the hypothalamus, frontal and temporal cortex (Mayer *et al.*, 1998). In rodents, there is some evidence that the *Sry* transcript is processed differently during development; circular transcripts are detected in the embryonic whole brain, but linear transcripts are produced in the postnatal brain (Mayer *et al.*, 2000). The functional significance of circular transcripts is not known; however, it has been suggested that the formation of circular RNA might reflect translational control, as very few circular *Sry* transcripts are loaded onto polysomes for translation (Capel *et al.*, 1993). Furthermore, this translation control of *Sry* in mice appears to be specific to tissue and developmental time points, and does not reflect a general switch from circular to linear transcripts, as *Sry* transcripts in the genital ridge are linear, but non-polyadenylated circular transcripts are detected in the adult testes (Capel *et al.*, 1993; Jeske *et al.*, 1995). Similar translation control has not been shown in humans; *Sry* transcripts exist as a linear, polyadenylated RNA in the adult human testes (Clepet *et al.*, 1993), and circular transcripts might not be possible in humans as there is no repeat structure around the *Sry* locus to allow for the formation of a stem loop structure and subsequent splicing to result in circular transcripts (Capel *et al.*, 1993).

In vitro and *in vivo* work has shown that *Sry* may act as a transcriptional activator at the promoters of genes encoding the enzymes TH (*TH*, Milsted *et al.*, 2004) and monoamine oxidase A (*MAOA*, Wu *et al.*, 2009). TH is the rate-limiting enzyme in dopamine biosynthesis (Cave & Baker, 2009), whilst monoamine oxidase A catalyses the oxidative deamination of a variety of monoamines including serotonin, adrenaline and dopamine (Shih *et al.*, 1999). Hence, Sry may have profound downstream effects on male-specific behaviours via influencing the catecholamine systems. SRY has also been shown to act as a transcriptional activator for members of the AP-1 transcription factor family, such as fos-related antigen 1 (Fra-1), by

binding onto *fra-1* HMG-box response element and activating *fra-1* transcription in cotransfection studies (Cohen *et al.*, 1994). It is possible that *Sry* binds to the promoter regions of numerous other brain-expressed genes which remain to be identified. In rats, *Sry* - in particular, the Sry3 locus[8] - has also been shown to regulate gene expression in renin-angiotensin system (Milsted *et al.*, 2010). A recent study in rats, in which expression of *Sry* was specifically downregulated in the substantia nigra region of the brain through oligonucleotide infusion, has provided data that brain-expressed *Sry* may directly influence neurochemistry and behaviour (Dewing *et al.*, 2006). In substantia nigra, where *Sry* gene expressed was downregulated, showed reduced levels of TH (which is consistent with the notion of *Sry* acting as an activator for *TH* transcription) but no alteration in neuronal number, whilst downregulation of *Sry* was also associated with motor deficits on the akinesia and limb-use asymmetry tests. The range of neurobiological parameters influenced by *Sry* may be amenable to testing using this rat knockdown model system.

Besides *Sry*, there are a number of other genes on the Y chromosome which are expressed in the brain (Kopsida *et al.*, 2009), such as *Sts* (Salido *et al.*, 1996), *Ddx3y* (also known as *Dbx1y*), *Ube1y, Kdm5d* (also known as *Smcy* and *Jarid1d*), *Eif2s3y, Uty* and *Usp9y* (Xu *et al.*, 2002), which could give rise to sex differences. Many of these have homologues on the X chromosome, but there is evidence that in many cases, the X and Y homologues are expressed to differing extents and/or in different brain regions; for example, whilst *Utx* is expressed highly in the amygdala, Y-linked paralogue *Uty* is expressed highly in paraventricular nucleus of the hypothalamus (Xu *et al.*, 2008).

(ii) X-linked gene dosage effects

Differential X-linked gene dosage between males and females can also underlie sexual dimorphism (Xu & Diestche, 2006). In mammals, females have two X chromosomes while males only inherit one; in order to balance this large potential imbalance in gene dosage between the sexes, one of the two X chromosomes in females is epigenetically modified and transcriptionally silenced through X chromosome inactivation (XCI; Valley & Willard, 2006). An important regulatory locus for XCI is the X inactivation centre (Xic), from where the noncoding gene *Xist* is expressed; *Xist* acts in *cis* to trigger silencing of the X chromosome (Heard & Disteche, 2006), with the downstream *Tsix* regulating its expression. *Xist* and *Tsix* are initially both expressed from the Xic; in differentiating female stem cells where XCI begins, *Tsix* RNA ceases to be expressed from one of the X chromosomes, leading to an accumulation of *Xist*

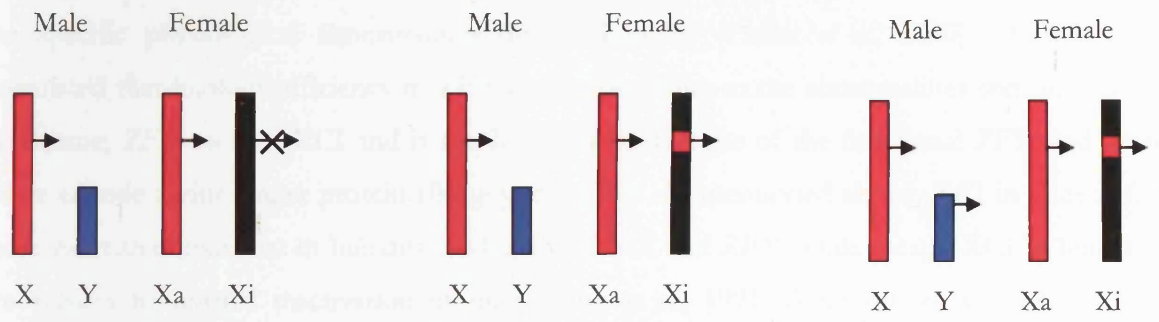
[8] There are multiple *Sry* gene copies in rats (Turner *et al.*, 2007), while most mammals only have one.

transcripts on that X chromosome, which becomes the inactive X (Willard & Carrel, 2001). The inactive X loses the histone modifications associated with active chromatin, and gains those linked with inactivity, such as hypoacetylation of histones H3 and H4 and methylation of H3 (Heard & Disteche, 2006). However, XCI is not complete; all genes in PAR1 (on the short tip) and most genes in PAR2 (on the long tip) escape XCI (Heard & Disteche, 2006). In humans, an estimated 15-20% of X-linked genes consistently escape inactivation, with a further 20% inactivated in some but not all cells (Carrel & Willard, 2005); the mechanism of inactivation escape might arise from failures to initiate or to maintain silencing (Brown & Greally, 2003). In mice, XCI is much more extensive, with only 13 genes known to escape XCI; these genes are not clustered together like in the case of humans (Yang *et al.*, 2010). Furthermore, eight of these genes also escape in human, suggesting a level of conservation. These escapees could potentially contribute to a gene dosage imbalance between the sexes, given that there are no functional homologues on the Y chromosome in males expressed in the similar manner as the X-linked homologue, i.e. comparable levels and pattern of expression in RNA transcripts, and additionally, similar functional homology in proteins, in the case of protein-coding genes (**Figure 1.2.3.1a**; Heard & Disteche, 2006).

Although the majority of X-linked genes that escape XCI have Y homology (Carrel & Willard, 2005), there is evidence indicating sex differences in expression levels of genes escaping XCI. Nguyen & Disteche (2006a) have noted a varied female-to-male ratio of 0.10 to 2.94 for 27 genes examined, suggesting differences do exist and may have functional consequences. In humans, the functional X-Y gene pair *PCDHX* (which escapes XCI; Lopes *et al.*, 2010) and *PCDHY*, in the cadherin gene superfamily and protocadherin subfamily, has slightly different amino acid sequences to each other, which might give rise to proteins with large differences in structure. *PCDHX* is predominantly expressed in the cerebellum and heart, whereas *PCDHY* transcripts are found in the kidney, liver, muscle, and testis; these different expression patterns in the brain and body suggest this gene pair might contribute to differences between the sexes (Blanco *et al.*, 2000). Xu and colleagues (2002) have examined six X-Y homologus gene pairs in mice and showed that in five instances, the expression of the Y homologue was not sufficient to compensate for the higher expression from the X homologue. Out of the six gene pairs, four have been known to escape XCI in either human or mouse (*Usp9x/y*, *Kdm5c/d*, *Eif2s3x/y*, *Utx/y*) and display higher mRNA expression in female than in male mice; additionally, some gene pairs were expressed in different spatio-temporal patterns (Xu *et al.*, 2002). Whereas *Usp9x/y* (Xu *et al.*, 2005) showed sexual dimorphic expression in both mRNA and protein levels, the sex differences in *Eif2s3x/y* mRNA levels were not preserved at the protein level (Xu *et al.*, 2006); in the same

vein, the sex differences of *Kdm5c/d* (Xu *et al.*, 2008a) and *Utx/y* (Xu *et al.*, 2008b) mRNA transcripts might not carry over to the protein level.

Figure 1.2.3.1a



In order to balance the single gene dosage from the X chromosome in males, one of the two X chromosomes in females is inactivated (Xi; black). The expression from both sexes is equal.

When a gene escapes XCI and expresses from the Xi, the gene dosage in females is higher than that in males. The gene expression is unequal and can underlie aspects of sexual dimorphism.

However, if there is a functional Y homologue expressed in males, then there are no differences in gene dosage between the sexes, even if the X-linked gene escapes XCI. The gene expression from both sexes is equal.

Figure 1.2.3.1a Schematic diagram explaining the possible gene dosage differences between the sexes in the case when an X-linked gene escapes XCI. Xa (pink): activated and expressed X chromosome; Xi (black): inactivated X chromosome.

Additional evidence for X-linked gene dosage effects can be seen in studies with sex chromosome aneuploidies, such as Turner Syndrome (TS) in humans. TS is a chromosomal disorder affecting approximately 1 in 2500 live female births, in which there is partial or complete loss of one X chromosome, and manifests a range of symptoms, for example, reproductive abnormalities, short stature and various neuropsychological deficits such as attentional problems, visuospatial processing impairments and social cognition problems (Lynn & Davies, 2007). The physiological and neuropsychological abnormalities are likely to be the result of haploinsufficiency (i.e. lacking of gene dosage) for one or more products of X-linked genes that normally escape XCI (Zinn & Ross, 1998). X-Y homologous gene pairs are good candidates for X-linked gene dosage effects; by definition, these gene pairs exist on both the X and Y chromosome in the male, and therefore the gene dosage for the male is two. Assuming that the gene dosage in the female is the same as that in the male, this will mean the X-linked gene will escape XCI, and in cases of chromosomal disorders such as TS, this X-linked gene could serve as a candidate for X-linked gene dosage effects. For example, the gene SHOX, located in PAR1 and thought to underlie bone growth and development, escapes XCI and its haploinsufficiency has been implicated in short stature in TS females (Ellison *et al.*, 1997; Rao *et*

al., 1997; Ross *et al.*, 2001). The X-Y homologous gene pair *RPS4X* and *RPS4Y* has also been suggested to underlie abnormalities in TS; interestingly, *RPS4X* is located in the long arm of the X chromosome, near the Xic, and is the only gene in the region to escape XCI in humans. The two genes encode isoforms of a ribosomal protein, whose aberrant levels might cause some of the specific physiological abnormalities observed in TS (Fisher *et al.*, 1990). It has been postulated that haploinsufficiency in *ZFX* could contribute to the abnormalities seen in Turner Syndrome; *ZFX* escapes XCI and is the X-linked homologue of the functional *ZFY*, and both genes encode a zinc finger protein (Burgoyne, 1989). As mentioned above, XCI in mice is far more extensive than that in humans, and both *RPS4X* and *ZFY*, while escape XCI in humans, are subject to normal inactivation in mice (Zinn *et al.*, 1991; Ashworth *et al.*, 1991). This difference in XCI between species could help explain the phenotypic differences between human TS and X-monosomy in mice (39,XO); the 39,XO mouse (an animal model for aspects of TS, see **section 1.2.4.1** for more details) are morphologically grossly normal and fertile, in contrast to human TS females who might display physiological and fertility problems (Lynn & Davies, 2007).

(iii) X-linked genomic imprinting effects

A third genetic influence on sexual dimorphism comes from X-linked genomic imprinting. Genomic imprinting is the epigenetic mechanism in which alleles are expressed and silenced depending on their parental origin; a maternally expressed imprinted gene will be expressed from the allele inherited from the mother and the paternally inherited allele will be silenced, and *vice versa* for a paternally expressed imprinted gene (Davies *et al.*, 2005b). Many imprinted genes are expressed in the brain (Davies *et al.*, 2006b) and therefore might play a significant role in cognition; a comprehensive table detailing brain-expressed imprinted genes can be found on the Behavioural Genetics Group website[9]. Recently, a study by Gregg and colleagues (2010b) found 1308 candidate imprinted loci using a genome-wide analysis of mouse brain tissues, and observed a preferential maternal expression of imprinted alleles in the developing brain and preferential paternal expression in the adult brain. Additionally, there are imprinted genes present on the X chromosome, with the potential to lead to sex differences (Davies *et al.*, 2006a). Sexual dimorphism arise as a consequence of the fact that males always inherit their single X chromosome maternally (as the Y chromosome must be inherited paternally, i.e. X^mY^p), whilst females inherit one X chromosome paternally and a second maternally (i.e. X^mX^p). Therefore, a maternally expressed X-linked imprinted gene may be expressed in both males and females (both

[9] http://www.bgg.cardiff.ac.uk/imprinted_tables/brain_table.html

possess X^m) whereas a paternally expressed X-linked imprinted gene may only be expressed in females, as they possess X^p but males do not (Davies *et al.*, 2006a). Furthermore, a maternally expressed X-linked imprinted gene may be expressed at a higher level in males than females, if the gene is subject to XCI (for example, *Xlr3b*; Davies *et al.*, 2005a); in females, the X^m, and therefore the maternally expressed X-linked imprinted gene, will be silenced 50% of the time (assuming non-skewed XCI). Another factor that can influence sex differences contributed from X-linked imprinted genes is the presence of functional Y homologue in males. **Table 1.2.3.1i** details the theoretical gene dosage for X-linked imprinted genes in males and females.

Table 1.2.3.1i shows theoretical gene dosages for X-linked imprinted genes in males and females, taking into account the direction of imprinting, XCI status and possibility of a functional Y homologue (adapted from Davies *et al.*, 2006a) Blue and pink cells are males and females respectively.

	Escapes XCI		Subject to XCI	
Imprinting direction	Functional Y homologue	No functional Y homologue	Functional Y homologue	No functional Y homologue
Paternally expressed	$X^mY^p = 1$	$X^mY^p = 0$	$X^mY^p = 1$	$X^mY^p = 0$
	$X^mX^p = 1$	$X^mX^p = 1$	$X^mX^p = 0.5$	$X^mX^p = 0.5$
Maternally expressed	$X^mY^p = 2$	$X^mY^p = 1$	$X^mY^p = 2$	$X^mY^p = 1$
	$X^mX^p = 1$	$X^mX^p = 1$	$X^mX^p = 0.5$	$X^mX^p = 0.5$

While there is evidence of X-linked genomic imprinting in humans, no genes have been formally identified (Davies *et al.*, 2006a). Effects of X-linked genomic imprinting have been shown in a seminal study on TS individuals by Skuse and colleagues (1997), in which it was demonstrated that 45,X^m performed worse than 45,X^p and 46,XX females on the Same/Opposite World task, which indexes behavioural inhibition. Regarding neuroanatomy, it has been shown in a MRI study that 45,X^m females showed a larger right hippocampal volume than 45,X^p subjects (Cutter *et al.*, 2006), which might contribute to the impaired memory retention of visuospatial information observed in 45,X^p females when compared to 45,X^m and 46,XX individuals in a Rey figure recall task (Bishop *et al.* , 2000). Kesler and colleagues (2003) has found larger right and left superior temporal gyrus volume in 45,X^m subjects than in 45,X^p and 46,XX females; the superior temporal gyrus is important in complex auditory stimuli and language processes, and it has been shown that 45,X^p females display superior verbal skills than 45,X^m individuals (Temple

et al., 1996; Skuse *et al.*, 1997). In functional neuroanatomy, there was preliminary evidence from a fMRI study that 45,X^m females displayed significantly more activation than 45,X^P subjects in the right superior and middle frontal gyrus (Tamm *et al.*, 2003).

In contrast to the lack of identified human X-linked imprinted genes, murine X-linked imprinted genes have been found (Davies, 2010). *Xlr3b*, *Xlr4b* and *Xlr4c* were found to be a cluster of X-linked imprinted genes (Raefski & O'Neill, 2005), with the maternally expressed imprinted gene *Xlr3b* postulated to be the candidate gene underlying parent-of-origin effects on cognition; 39,X^mO, 39,X^PO and 40,XX mice (i.e. animals generated from the 39,XO mouse model, see **section 1.2.4.1** for more details) were tested on a 2-choice visual discrimination task with reversal, in which 39,X^mO animals specifically performed worse than 39,X^PO and 40,XX females during reversal (Davies *et al.*, 2005a). Davies and colleagues tested the XO mouse model on this particular 2-choice visual discrimination task with reversal, because Skuse and colleagues (1997) had observed poorer performance in behavioural inhibition (from the Same-Opposite World task) from 45,X^m Turner Syndrome girls, compared to 45,X^P and 46,XX girls. However, in Skuse's study, there was a possible confound of cryptic mosaicism in the Turner Syndrome girls; this particular confound was eliminated with the use of XO mouse model (see Section **1.2.4.1**). *Xlr3b* was expressed throughout the brain, including frontal cortex and hippocampus, which suggests a possible role in cognition; however, the exact brain function of the gene is unknown. *Rhox5* is another X-linked imprinted gene in mice and is a member of a HMG cluster that is predominantly expressed in reproductive tissues, and thus *Rhox5* might have a role in the development of these tissues. Interestingly, whilst it was expressed from paternally inherited allele prior to embryonic day 7.5 at the blastocyte stage, its expression switched to that from the maternally inherited allele thereafter, and finally, biallelic expression was observed in adult brain (Kobayashi *et al.*, 2006; Davies, 2010). Recently, another X-linked imprinted gene, *Fthl17*, has been found; this gene is expressed from very early on in development, at the 2-cell stage, from the paternally inherited allele (Kobayashi *et al.*, 2010). Fthl17 gene possesses a metal-binding motif, but exact function is unknown.

1.2.4 Utility of mouse models

In general, mice represent a good experimental system for modelling human brain conditions as a large number of matched subjects can be tested in a controlled environment and the behavioural and drug history of subjects can be carefully regulated. The physiology (and specifically neurobiology) of mice can be intimately examined, making it particularly amenable for investigating the brain and underlying cognition, and the mouse genome has been extensively

mapped and may be readily manipulated to create mutant and transgenic mice. Furthermore, a large number of behavioural assays exist for mice, including those that test complex cognitive constructs (Humby & Wilkinson, 2006; Isles *et al.*, 2003). The degree to which gene content and order in man and mouse is considerably conserved, with substantially detailed comparative maps between the two species (Carver & Stubbs, 1997); however, the mouse chromosomes have undergone many genomic rearrangements over evolutionary time and are subject to different evolutionary pressures, and so caution will need to be taken when comparing between man and mouse. For example, the X-linked imprinted gene in mouse, *Xlr3b*, does not have a human orthologue (Raefski & O'Neill, 2005) and so findings regarding its imprinting status and associated cognitive effects would need to be majorly reinterpreted for the case of humans. Also, there is a large discrepancy in the number and identity of genes that escape XCI between man and mouse (Yang *et al.*, 2010).

1.2.4.1 39,XO mouse model

Turner Syndrome can be used to investigate the effects of X-linked imprinting and gene dosage on brain and behavioural phenotypes, and in the same vein the 39,XO mouse model could also shed light on these imprinting and haploinsufficiency effects. Using two separate crosses, 39,XO mice could be engineered such that they inherit their single X chromosome either maternally or paternally, resulting in the karyotypes $39,X^{m}O$ and $39,X^{p}O$, respectively. Furthermore, the 39,XO mouse model does not have some of the confounds which are inherent when examining TS females; for example, cryptic mosaicism (i.e. where one of the X chromosome might be partially, rather than wholly, lost, and additionally Y chromosome material might be present) is a potential confound in human TS studies. The 39,XO mice are generally healthy with no gross morphological abnormalities and are fertile, so that any neurobiological or behavioural findings are unlikely to be confounded by growth or hormonal problems. It would appear that the 39,XO mouse model can recapitulate some of the phenotypes observed in TS females, in that the animals do show some minor abnormalities reminiscent of TS, including high frequency hearing loss and aberrant cochlear architecture (Hultcrantz *et al.*, 2000) and reduced thyroid activity and body temperature (Deckers & Van der Kroon, 1981; Deckers *et al.*, 1981). Moreover, $39,X^{p}O$ mice are developmentally retarded relative to their 40,XX siblings early in development (Thornhill & Burgoyne, 1993), whilst both $39,X^{p}O$ and $39,X^{m}O$ groups exhibit some degree of postnatal growth retardation (Burgoyne *et al.*, 2002). With regard to behavioural phenotypes, both 39,XO mice and TS individuals exhibit deficits in fear reactivity (Rovet & Ireland, 1994; Isles *et al.*, 2004), attention (Romans *et al.*, 1998; Davies *et al.*, 2007) and behavioural inhibition

(Skuse *et al.*, 1997; Davies *et al.*, 2005a). The above data would suggest that the 39,XO mouse model has a reasonable level of face validity in modelling aspects of TS.

However, there are disadvantages with the 39,XO mouse model. It is possible that, as a consequence of the more extensive XCI of the mouse X chromosome (i.e. fewer X-linked genes escape XCI in mice), with only 13 genes currently known to escape XCI (Yang *et al.*, 2010), X-monosomy effects observed in man (with less extensive XCI) would be absent or substantially less severe and therefore cannot be readily modelled in the mouse. There are also differences between human and mouse in terms of gene content (e.g. *Xlr3b* is only present in the mouse and not in man, Raefski & O'Neill, 2005).

Another issue is the fact that, unavoidably, two separate crosses (details below) were required to generate the 39,X^mO and 39,X^PO (and 40,XX littermates) animals (Burgoyne & Evans, 2000; Evans & Phillips, 1975), and thus, any behavioural differences found between 39,X^mO and 39,X^PO mice might be influenced by differential pre-weaning maternal-offspring and/or offspring-offspring interactions. A further possible confound arises from the presence of the *Paf* mutation in the father utilised in 39,X^mO generating cross, which is not present in the mother in the 39,X^PO generating cross; this would result in different littermates (some are *Paf* heterozygotes, some not) and possibly different inter-littermates interactions. In order to help control for this, the *Paf* mutation was introduced in the mother in the 39,X^PO cross, so that both crosses produce *Paf* heterozygote littermates, 40,XX^{Paf} (from the 39,X^mO cross) and 40,$X^{Paf}X$ (from 39,X^PO cross). This strategy tackles the possible confound to a degree; however, in 40,XX^{Paf}, the *Paf* mutation is inherited paternally whereas in 40,$X^{Paf}X$, the mutation is inherited maternally, and so there is a possibility that the two *Paf* heterozygote littermates are not identical due to unanticipated *Paf* parent-of-origin effects.

(i) Generation of 39,X^mO mice

39,X^mO mice may be generated at an efficient rate (~40% of female offspring are 39,X^mO) using the cross described by Burgoyne and Evans (2000). This cross involves the mating of male mice of karyotype 40,$X^{Paf}Y^*$ with female mice of karyotype 40,XX. *Paf* (Patchy fur) is a semidominant X-linked mutation which maps close to the distal end of the murine X chromosome, near the pseudoautosomal region (PAR; Lane and Davidsson, 1990), and Y* is a variant Y chromosome with a compound PAR, flanked at the distal end by an X PAR boundary and other X-specific material (including the wild type *Paf* allele). The Y* variant retains its Y centromere, which is inactive.

During male gametogenesis, two types of recombination orientations, 'parasynapsed' and 'staggered', are possible. The parasynapsed recombination orientation can lead to formation of large dicentric and acentric chromatids, which are subsequently lost in anaphase II, resulting in sex chromosome-null (or 'O') gametes. These gametes may fertilise a normal female gamete to produce 39,$X^{m}O$ female progeny. The parasynapsed orientation may also produce gametes containing a non-recombinant X^{Paf} chromosome which may fertilise a normal female gamete to produce 40,XX^{Paf} female offspring. Lastly, gametes containing Y* products might be formed which may fertilise a female gamete to lead to XY* male progeny.

In the staggered formation, male gametes containing either the X^{Paf} or Y^{*X} chromatids may be formed to fertilise maternal gamete to produce 40,XX^{Paf} and 40,XY^{*X} female offspring. Additionally, male progeny may be produced by fertilisation of maternal gametes with sperm containing X^{PafY*} or Y* chromatids.

The presence of the *Paf* mutation increases the crossing over in the parasynapsed orientation and hence, increases the rate at which 39,$X^{m}O$ mice are produced. Technical details of the 39,$X^{m}O$ generating cross are found in **Figure 1.2.4.1a**.

(ii) Generation of 39,$X^{P}O$ mice

39,$X^{P}O$ mice may be generated from the cross described by Evans and Phillips (1975). This cross involves the mating of normal 40,XY male mice with female mothers that are heterozygous for a large paracentric chromosomal inversion In(X)1H. However, the cross used to produce offspring for the research of this thesis was slightly modified; mothers of karyotype 40,In(X)1H/X^{Paf} were used (instead of the 40,In(X)1H/X used in the Evans and Phillips cross). The *Paf* mutation on the second X chromosome is not necessary for the production of the 39,$X^{P}O$ offspring, but it was introduced into this cross to address the fact that the generation of the 39,$X^{m}O$ mice involved the introduction of the *Paf* mutation and generated 40,XX^{Paf} littermates, whereas the generation of the 39,$X^{P}O$ cross, if unmodified, would produce 40,XX siblings (see caveats described earlier).

The large In(X) inversion promotes crossing over between the chromosomes during gametogenesis in the mother. In the situation where the crossing over occurs within the inversion, dicentric and acentric chromatids may be produced, resulting in 'O' gametes after the subsequent loss of these chromatids in anaphase II. 'O' female gametes may be fertilised by a male gamete containing either a single X or a single Y chromosome to generate 39,$X^{P}O$ and 39,OY offspring. 39,OY offspring dies prenatally. Additionally, X^{Paf} chromatids may be

produced, and when fertilised by a male gamete containing either a single X or a single Y, 40,$X^{Paf}X$ and 40,$X^{Paf}Y$ (latter not tested in this thesis) may be generated respectively.

In the alternative situation where the crossing over occurs between the *Paf* mutation and the inversion, a recombinant X product may result, and when fertilised by a male gamete containing either a single X or a single Y, 40,XX and 40,XY (latter not tested in this thesis) may be generated respectively. A X^{Paf} chromatid product may be generated, which will produce 40,$X^{Paf}X$ and 40,$X^{Paf}Y$ offspring as in the first crossing over situation. Technical details of the 39,$X^{P}O$ generating cross are found in **Figure 1.2.4.1b**.

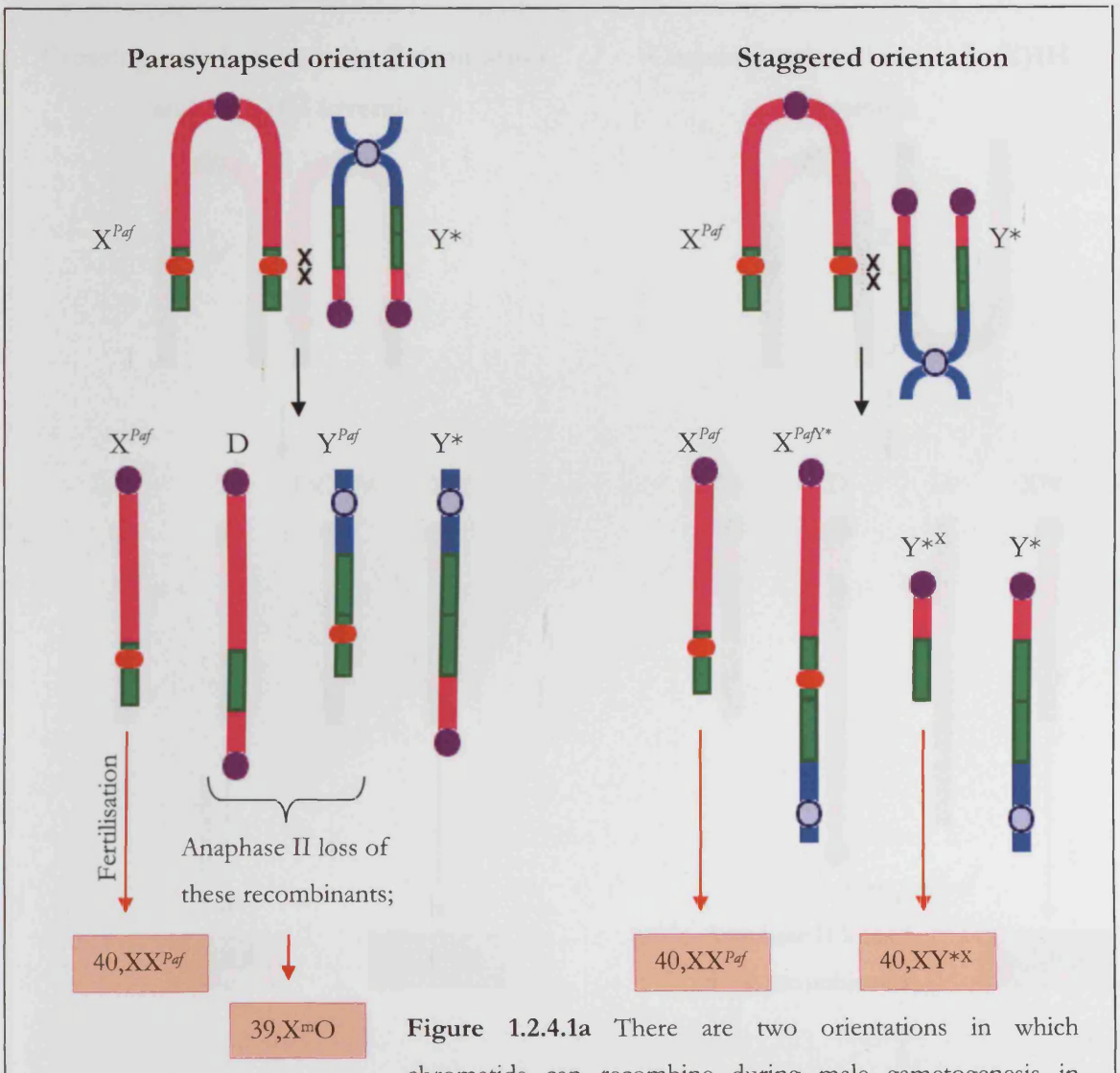


Figure 1.2.4.1a There are two orientations in which chromatids can recombine during male gametogenesis in 40,X^{Paf}Y*. The parasynapsed orientation can result in a dicentric (D) or an acentric Y^{Paf} chromosome (functionally acentric due to inactive Y centromere). Subsequent loss of these chromosomes during anaphase II may result in sex chromosome-null or 'O' gametes. A non-recombinant X^{Paf} chromosome may also be produced. In the staggered formation, X^{Paf} chromosomes, along with Y*^{X} chromosomes, may be generated. If an O, X^{Paf}, Y*^{X} sperm fertilises a normal gamete from a 40,XX mother, the following offspring, 39,X^{m}O, 40,XX^{Paf}, 40,XY*^{X}, may be generated respectively. Note that the diagram does not show the rarer recombinant chromosomes, and only displays female offspring that was utilised in this thesis.

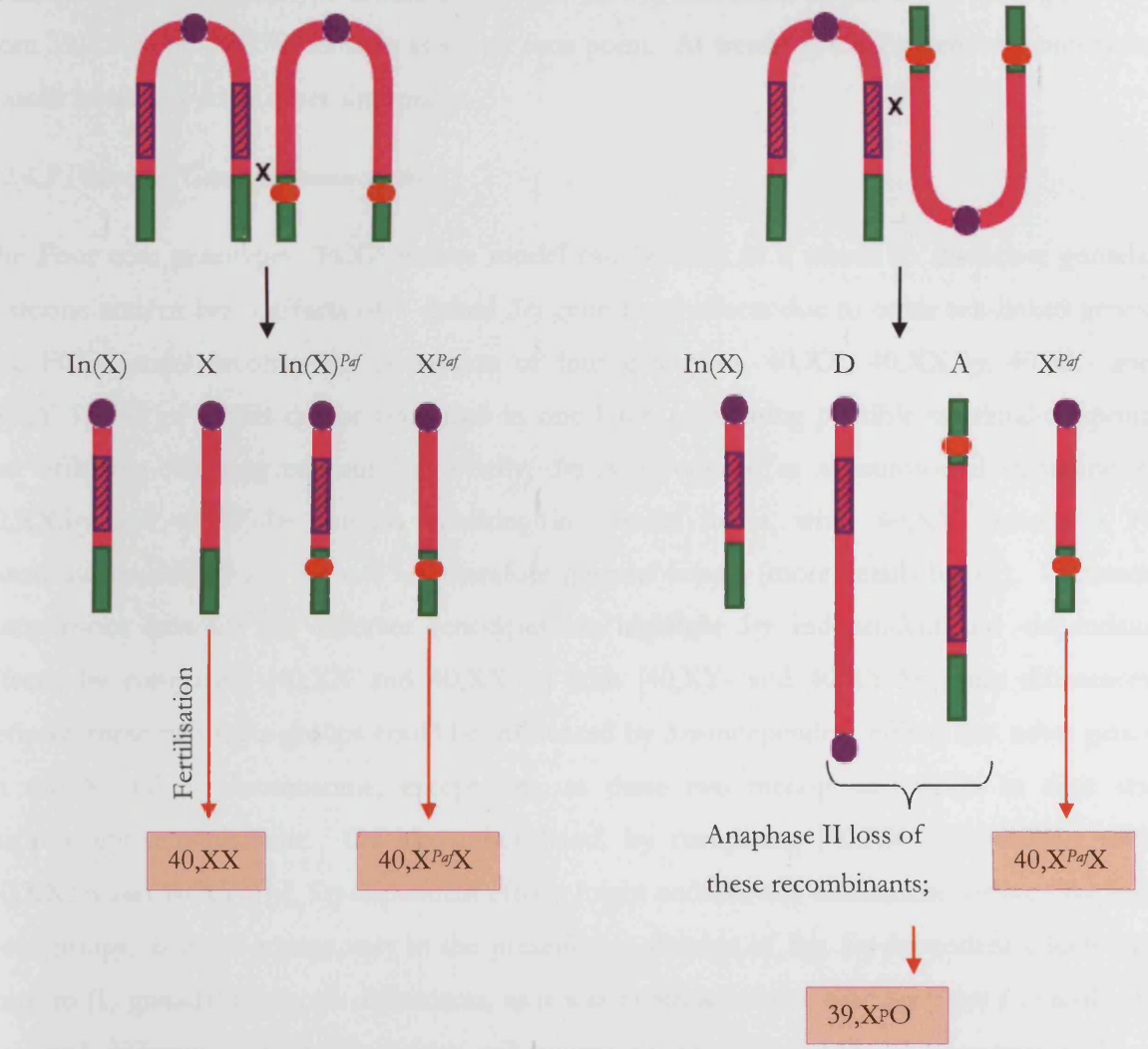


Figure 1.2.4.1b During gametogenesis in In(X)1H/X^{Paf} mothers, if the crossing over occurs between *Paf* mutation and the inversion, recombinant X or X^{Paf} chromosomes may be generated. If crossing over occurs within the inversion, a large dicentric (D) or an acentric (A) chromosome may be formed; the subsequent loss of these recombinants may result in sex-chromosome null or 'O' gametes. A non-recombinant X^{Paf} chromosome may also be produced. If an O, X or X^{Paf} egg is fertilised by a paternal X-containing sperm, the following offspring, 39,X^{P}O, 40,XX , 40,X^{Paf}X, may be generated respectively. Note that the diagram only displays female offspring that were utilised in this thesis.

The *Paf* heterozygotic littermates were identified at seven to nine days of age, at which time the *Paf* animals sport the most pronounced patchy fur phenotype and appear nude; as the animals become older, this phenotype diminishes and so the *Paf* littermates might not be distinguishable from 39,$X^{m}O$ and 39,$X^{p}O$ females at a later time point. At weaning, the *Paf* heterozygotes were housed separately from other littermates.

1.2.4.2 Four Core Genotypes mouse model

The Four core genotypes (FCG) mouse model can be used as a means to dissociate gonadal hormone and/or brain effects of Y-linked *Sry* gene from effects due to other sex-linked genes. The FCG model involves the generation of four genotypes, 40,XX, 40,XX*Sry*, 40,XY- and 40,XY-*Sry*, all of which can be produced in one litter, eliminating possible maternal-offspring and offspring-offspring confounds. Briefly, *Sry* is introduced as an autosomal transgene in 40,XX*Sry* and 40,XY-*Sry* animals, resulting in gonadal males, while 40,XY- possess a Y-chromosome deleted for *Sry* and are therefore gonadal female (more details below). Different comparisons between the different genotypes can highlight *Sry* -independent and -dependent effects; by comparing [40,XX and 40,XX*Sry*] with [40,XY- and 40,XY-*Sry*], any differences between these two meta-groups could be influenced by *Sry*-independent effects (i.e. other genes on the X and Y chromosome, except *Sry*), as these two meta-groups differ in their sex chromosome complement. On the other hand, by comparing [40,XX and 40,XY-] with [40,XX*Sry* and 40,XY-*Sry*], *Sry*-dependent effects might underlie any differences between the two meta-groups, as these groups vary in the presence or absence of *Sry*; *Sry*-dependent effects can point to (1) gonadal hormone differences, as it was explained above that *Sry* plays a crucial role in gonad differentiation and therefore, subsequent production of gonadal hormones such as testosterone, and/or (2) brain effects of *Sry*, as it has been shown that *Sry* is expressed in the brain and therefore might influence behaviour and cognition. **Figure 1.2.4.2a** describes the two comparisons.

Whilst undoubtedly useful, this model is not without its caveats; while normal 40,XX females are generated within the model, normal 40,XY males cannot be produced. 40,XY-*Sry* animals might superficially resemble normal 40,XY males, but it has been shown that 40,XY and 40,XY-*Sry* males are not identical in some aspects of behaviour and neurobiology, such as latency to thrust in mating test, total visits and number of visits to a male stimulus animal in social exploration test, and the number of TH-ir neurons in the anteroventral periventricular nucleus of the preoptic region (De Vries *et al.*, 2002).

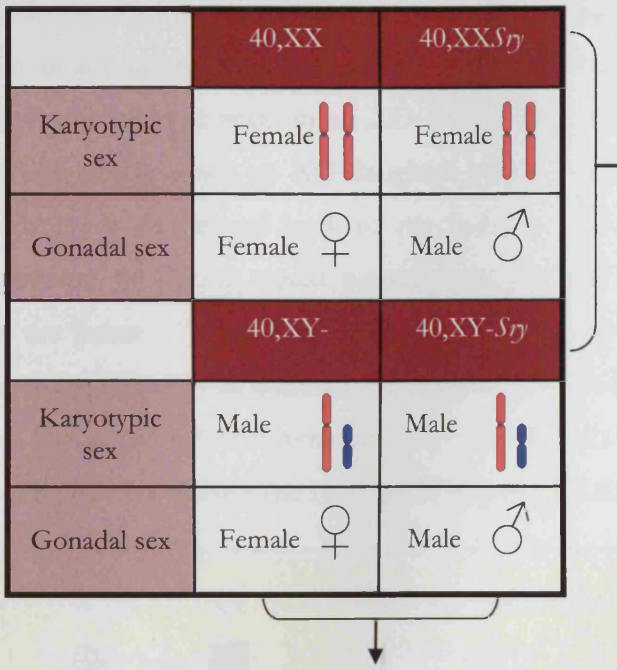


Figure 1.2.4.2a Diagram outlining the possible differences within the FCG mouse model and the associated effects, either *Sry*-independent or *Sry*-dependent. The statistical design of Two Way ANOVA utilised in the experimental chapters with Between Subject factors of SEX CHROMOSOME COMPLEMENT (*Sry*-independent) and GONADAL SEX (*Sry*-dependent) was used to reveal any differences.

The Y- chromosome possessed by some animal in the FCG model is a variant of Y^{129} derived from the mouse strain 129, deleted for the testis-determining gene *Sry* (*Tdy*^{m1} mutation, Lovell-Badge and Robertson, 1990). XY^{tdym1} males (referred to as XY-*Sry* males in this thesis) were originally generated on an MF1 background at MRC National Insitute for Medical Research, UK in the laboratory of my collaborator Dr Paul Burgoyne (Mahadevaiah et al., 1998), by introducing an *Sry* transgene derived from the transgenic line C57BL/6Ei-$Y^{AKR/J}$TgN(Sry-129)2Ei into pronuclear stage embryos from XY^{tdym1} females (referred to in this thesis as XY- mice); Y^{tdym1} is a 14kb deletion in the short arm of the Y chromosome which removes *Sry* (Lovell-Badge & Robertson, 1990; Gubbay *et al.*, 1992). *Sry* is introduced as a fully penetrant transgene inserted onto an autosome; the location of the insertion and the copy number of the

transgene is currently not known. Parents of the cross possessed the so-called 'uniform X' chromosomes in an attempt to reduce variability in the data; the uniform X chromosome stock was bred by mating a wildtype male to an XO female, both from a random bred MF1 strain background, in order to generate an XO daughter with a paternal X (**Figure 1.2.4.2b**). This particular XO daughter was mated back to the father so that the XX females (uniform X chromosome females) produced would possess two identical X chromosomes which both originated from the father. The uniform X chromosome was introduced into the present FCG cross by mating males (XY-*Sry*) to uniform X chromosome females, resulting in XY-*Sry* progeny which possessed the uniform X chromosome, and those animals were in turn bred with uniform X chromosome females to produce the FCG cross (**Figure 1.2.4.2b**).

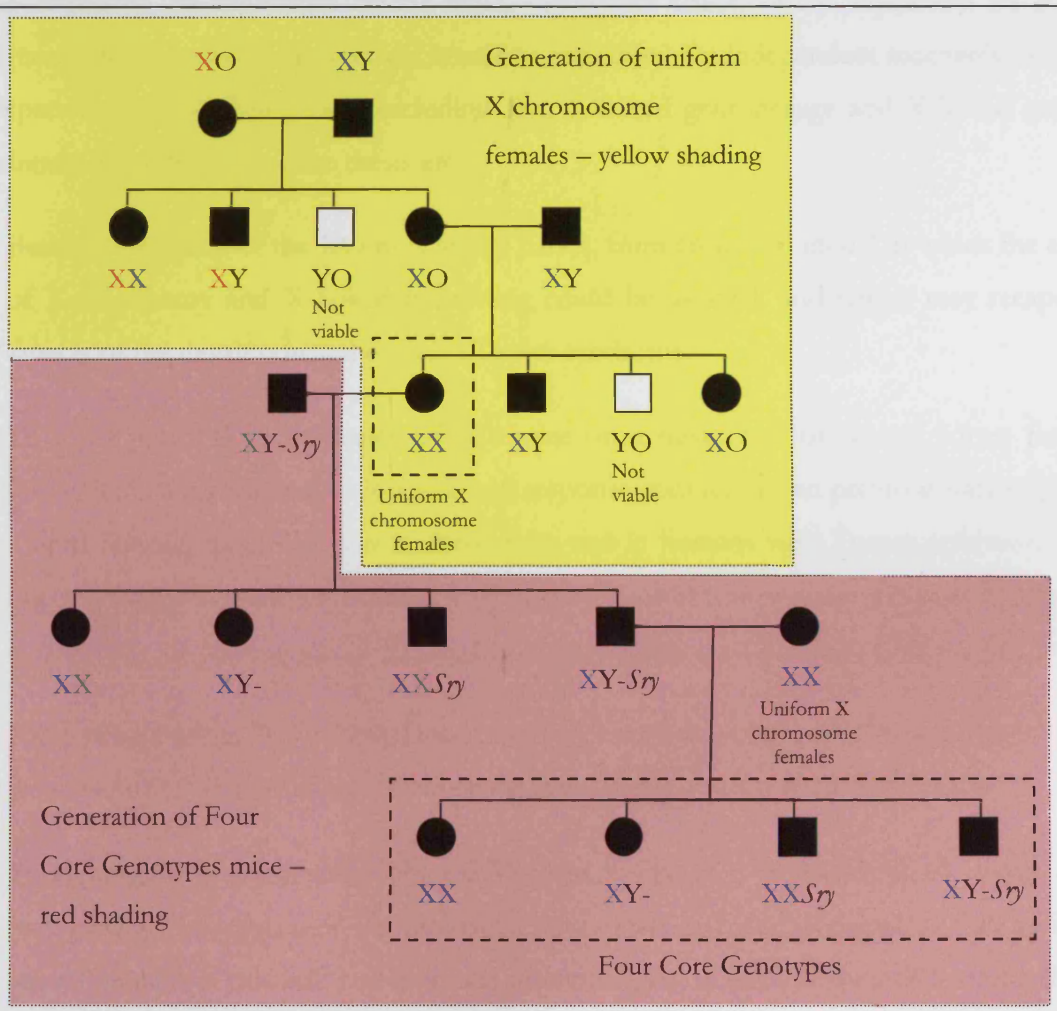


Figure 1.2.4.2b Details of uniform X chromosome females and Four Core Genotypes generation crosses. Black filled circles and squares denote gonadal females and males respectively.

One advantage of the FCG model is that all four genotypes are produced within any one litter, thus negating the possibility of inter-litter effects: 40,XX (gonadal females, karyotypic females),

40,XY- (gonadal females, karyotypic males), 40,XX*Sry* (gonadal males, karyotypic females) and 40,XY-*Sry* (gonadal males, karyotypic males); hereafter referred to as XX, XY-, XX*Sry* and XY-*Sry* respectively. Subjects were first distinguished by external genitalia (gonadal sex), housed according to their gonadal sex, and then genotyped according to the methods described in Chapter II, **2.9.2.**

1.3 Aims of the thesis

The main aim of the thesis is to address the extent to, and the specificity with which, *Sry*-dependent and *Sry*-independent mechanisms might influence aspects of brain function and behaviour using two well-characterised mouse models. *Sry*-dependent mechanisms comprise direct effects of *Sry* expression on the brain, or indirect effects of *Sry* expression on gonadal differentiation and subsequent gonadal hormone secretion. *Sry*-independent mechanisms consist of expression of Y-linked genes (excluding *Sry*), X-linked gene dosage and X-linked genomic imprinting. Specific aims of the thesis are:

- Basic phenotyping of the XO mice in my hands, from an animal model in which the effects of X-monosomy and X-linked imprinting could be assayed, and which may recapitulate aspects of the developmental disorder Turner syndrome.

- To investigate the performance of XO mice on a novel task of frontal cortex function (biconditional discrimination learning and response conflict), given previous data suggesting altered frontal cortex function in these mice and in humans with Turner syndrome. One might tentatively predict that there might be an X-linked imprinting effect in the response conflict part of the task (given the findings from Davies *et al.*, 2005a and Skuse *et al.*, 1997).

- Basic phenotyping of the Four Core Genotypes mouse model in my hands, in which *Sry*-dependent and -independent effects on brain and behaviour could be dissociated.

- To investigate performance of the FCG mouse model on a second assay of frontal cortex functioning (two-way visual discrimination with reversal), based on previous data indicating sex differences in this task and X-linked imprinted gene effects on reversal learning (Davies et al., 2005a). The aim of this task was to see the extent of gonadal hormones and sex chromosome complement contributes to task performance, given that both gonadal hormones and sex chromosome mechanisms have been shown to play a role in reversal learning.

- To investigate whether any identified *Sry*-dependent effects on brain function and behaviour were likely to be direct or indirect (i.e. hormonally-mediated) through assaying *Sry* gene expression in the brain and systemic testosterone levels.

- To describe the possible relevance of experimental findings to sex differences in brain and behaviour in rodents and man, and to sex chromosome disorders such as Turner syndrome.

Chapter II

General Materials and Methods

This chapter describes routine procedures that were carried out during the course of research for this thesis and descriptions of any experimental apparatus used. All procedures involving live animals were performed in accordance with the guidelines and requirements set out in the U.K. Animals (Scientific Procedures) Act (1986) and in the Home Office Project Licence granted to Professor Lawrence Wilkinson (PIL 80/1937). Work was performed under the Home Office Personal Licence granted to Phoebe Lynn (PIL 30/7662).

2.1 Subjects

2.1.1 XO mice

XO mice on a MF1 random bred albino background were generated at the MRC, NIMR, Mill Hill, London by my collaborator Dr Paul Burgoyne using the crosses as described in the General Introduction (Burgoyne & Evans, 2000; Evans & Phillips, 1975; additional details of the crosses are given in Experimental Chapter III, **3.2.1**). Experimental groups of XO mice, together with their female siblings, were transported to Cardiff at age of five months and kept in isolators for treatment of *Pasteurella pneumotropica* infection and then housed in the animal house of the School of Psychology, Cardiff University at 2-4 mice per cage.

2.1.2 Four Core Genotypes mice

The Four Core Genotypes mice (FCG) used in this thesis were originally created by my collaborator Dr Paul Burgoyne in the MRC, National Institute for Medical Research (NIMR) Mill Hill London on a MF1 random bred albino strain background using the crosses as outlined in the General Introduction (Lovell-Badge and Robertson, 1990; Mahadevaiah *et al.*, 1998; additional details of the crosses are given in Experimental Chapter V, **5.2.1**). A colony was established at the Babraham Institute, Cambridge, UK when the Behavioural Genetics Group was located there (prior to 2007). For the experiments in this thesis, the lines were established at Cardiff from animals transported from the Babraham Institute to the animal house in the School

of Psychology, Cardiff University. For the purposes of housing, male and female mice were defined according to gonadal status and were group housed separately, with 2-5 mice per cage.

2.1.3 XY wildtype mice

Additionally, for the generation of wildtype 40,XY males (hereafter referred to simply as wildtype XY), a separate cross was set up in the animal house at the School of Psychology, Cardiff University with wildtype MF1 males and wildtype MF1 females with uniform X chromosome (see General Introduction for more information on uniform X chromosome and Chapter VI, **6.2.1** for details of the wildtype XY generating cross). All female progeny of the cross were culled. Wildtype XY males were housed together with littermates in groups of 2-5.

2.2 General animal husbandry

All experimental animals were group housed in environmentally enriched cages (with cardboard tubes) in a temperature and humidity controlled holding room (21±2°C and 55±10% respectively), with a 12-hour light-dark cycle (0700 lights on/1900 lights off). Standard mouse chow (rodent maintenance feed, SDS, U.K.) and water were available *ad libitum* unless stated otherwise (in some cases restriction schedules were used to motivate performance in behavioural tests, see later). During the course of an experiment, the home cages were cleaned by the experimenter once a week at the same time and on the same day of the week, in order to minimise any disruption to the experiments.

All experimental animals were regularly monitored for signs of ill health; any mice which appeared unwell were immediately assessed by the NACWO animal technician and the vet. Weights were measured routinely and on a more frequent basis during restriction schedules. Sentinel mice housed in the holding room were regularly assayed for pathogens at Harlan, U.K.

2.3 Handling and body weight measurement

Imported animals were allowed at least two weeks to habituate to the new holding room environment at Cardiff University. All animals were handled once every day for two weeks (approximately two minutes per animal per day) prior to behavioural testing. Body weights were measured regularly, and weights were recorded at the same time each day (around 1700, following behavioural testing, and if animals were on water restriction, following two hours of free access to water).

2.4 Determination of oestrous

Vaginal smearing was performed with a cotton wool swab, to determine the oestrus status of the animal. Cells were smeared on cleaned slides and stained in 0.05% cresyl violet solution for around seven minutes. This quick procedure of vaginal smearing did not appear to cause the mice any discomfort. Smearing was performed daily for ten days, before the start of behavioural testing, in order to establish an oestrous cycle pattern. The procedure was then repeated at particularly important stages of behavioural testing and after the completion of testing. Smears were examined under the 10x magnification of a light microscope; stage of oestrous (dioestrous, proestrous or oestrous) could be determined based upon the morphology of cells in the sample (**Figure 2.4a**; Murr *et al.*, 1973).

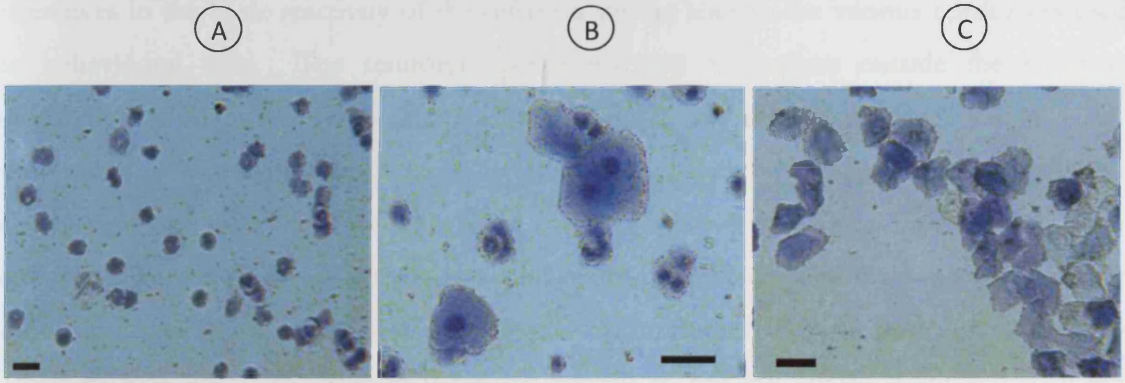


Figure 2.4a
A: diestrous stage, characterised by a mucosal discharge containing small, dense leucocytes.
B: proestrous stage, characterised by larger, nucleated epithelial cells.
C: oestrous stage, characterised by many large cornified cells with degenerate nuclei.
Bar = 10μm. Photos courtesy of Dr William Davies

2.5 Altered home cage water availability protocol

Animals were placed on a water restriction protocol two weeks before the start of any behavioural testing that utilised a liquid reward as a task reinforcer. This was done as a well established means within the laboratory of motivating performance in the tasks (Isles *et al.*, 2004; Davies *et al.*, 2005a). The mice were allowed access to home cage water for four hours per day during the initial two days of the protocol, and then two hours per day thereafter and throughout the testing period. For the initial seven days of water restriction, water bottles were weighed at the start and end of water access to ensure that animals were drinking sufficiently during that period. Animals were given a full 24 hours of free water access at least once every four weeks and then placed back on the restriction schedule until the end of testing. Body weights of

animals were routinely monitored to ensure that subjects remained above 85% of their free-drinking weight. Any subjects with weights below 80% of their free-drinking weight and/or showing signs of dehydration were immediately given *ad libitum* water access for at least 12 hours. Standard mouse chow was available *ad libitum* during the water restriction protocol.

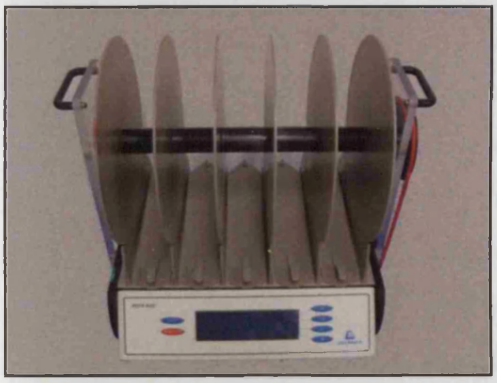
2.6 Reinforcer (reward) preference test

Prior to any behavioural testing, and following the stabilisation of body weight on the water restriction schedule, the animals were habituated, where appropriate, to the liquid reinforcers (rewards) used in many of the appetitively motivated behavioural tasks, which was either 10% condensed milk (Nestlé, U.K.), 20% sucrose, or 10% grape-flavoured maltodextrin, solutions. This general test was necessary in order to exclude the possibility that there were pre-existing differences in the basic reactivity of the different mouse lines to the various reinforcers used in the behavioural tests. The reinforcer preference test took place outside the home cage environment in eight test cages (285 × 130 × 120mm, one mouse per cage) during daily 10-minute sessions over a period of six consecutive days. These test cages contained no sawdust bedding and had two Velcro squares glued to the bottom of the cage, onto which two small containers (diameter 25mm) containing liquid could be affixed. The containers were weighed before and after each session to assess fluid consumption. For the initial two days, both containers held water; this was to habituate animals to the test environment and to measure general water consumption. For the remaining four days, one container held water while the other held the reinforcer. The positions of the containers were switched between days to avoid positional response bias. In all cases, the mice were provided with excess volume of fluids. Reinforcer preference was defined as the amount of reinforcer consumed as a percentage of the total amount of liquid when given the choice between water and reinforcer over the four days of testing.

Note that the general procedure outlined above was for testing one reinforcer against water. Due to task design, for the biconditional discrimination testing with XO animals, two reinforcers were used in the experimental paradigm. The above general procedure was slightly modified for this purpose and full details are outlined in Chapter IV, **4.2.3**.

2.7 Behavioural apparatus

2.7.1 Rotarod



The basic motoric competence of the subjects was tested on the rotarod (Ugo Basile, Italy), which consisted of a rotating bar with a non-slip surface (25mm in diameter). The rotating speed and acceleration of the bar could be changed. The bar was separated into five segments (each 55mm wide) with rotating partitions (120mm in diameter). One mouse was put on a given segment and a lever underneath was raised to start a time counter. When the mouse fell and hit the lever down, the counter was stopped and the time spent on the bar was recorded.

2.7.2 Locomotor activity boxes

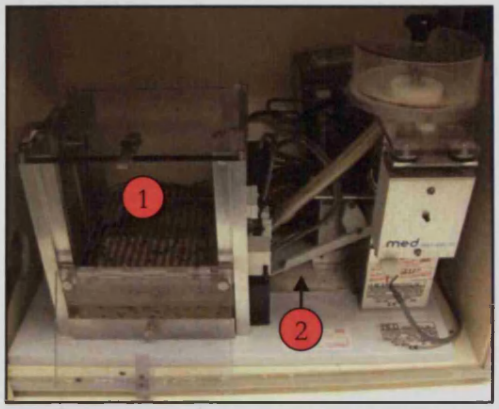
Locomotor activity was measured using clear Perspex boxes made in-house (each measuring 215 × 360 × 200mm, width × length × height); testing was performed in a battery of 12 boxes, with one subject run in each box. Two infra-red beams spanned each box, located at 30mm from each end of the box and 10mm from the floor of the box. Air holes were present on the top side of the box. Infra-red beam breaks data were recorded by a computer with custom written BBC BASIC V6 programmes (Cambridge Cognition Ltd., Cambridge, U.K.).

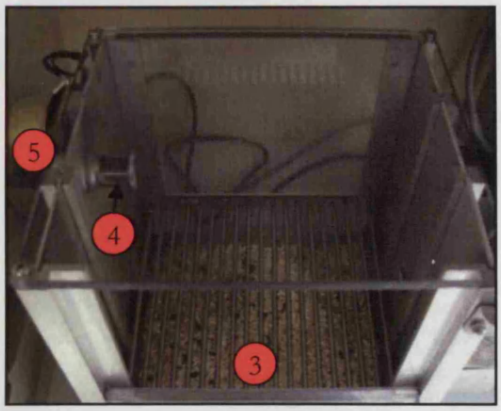
2.7.3 Elevated zero maze

Fear reactivity was measured using the elevated zero maze (Shepherd *et al.*, 1994; Cook *et al.*, 2002). The maze (600mm in diameter), elevated 500mm above the floor, was constructed with silver painted metal, which was subsequently covered with black tape for Ethovision tracking purposes. The maze comprised of two open quadrants and two closed quadrants with an open roof and high walls (220mm high), and was illuminated by a 40W red light giving an overall light intensity of ~5 lux. Subjects were tracked using the Ethovision tracking system (Noldus, U.K.).

2.7.4 Biconditional discrimination and 'Stroop' tasks

The biconditional discrimination and 'Stroop' tasks (which were carried out sequentially using the same basic apparatus with different stimuli configurations) were conducted in a standard operant chamber (**Figure 2.7.4a**; Med Associates Inc., U.S.A.). The operant test chamber (140mm × 160mm × 120mm, length × width × height) consisted of two nose poke response apertures, two stimulus lights and a food magazine at the right wall, and set into the left wall were a loudspeaker (2900Hz, 65dB) and a house light (2.8W). The bottom of the chamber was either a grid floor (19 stainless steel rods, 48mm in diameter) or a smooth Perspex floor, over a removable litter tray. Behind each nose poke response hole (13mm in diameter, 10mm in depth) was set a yellow LED with an infra-red beam, which detected nose poke responses when the animal's nose was 6.4mm into the aperture. The stimulus lights (7.9mm in diameter) above the nose poke holes were bright yellow LEDs. The food magazine (51mm × 51mm, height × width) had a hole in the bottom, through which a small stainless steel cup attached onto the dipper arm could deliver the reinforcer (20% sucrose solution or 10% maltodextrin solution, 0.01ml in volume). The chamber was installed within a melamine high density wood cubicle to block out external noise; additionally, a fan provided ventilation and constant background noise. A computer running custom written programmes with the MED-PC IV software (Med Associates Inc., U.S.A.) on Windows XP platform was used to operate the chamber and record data.





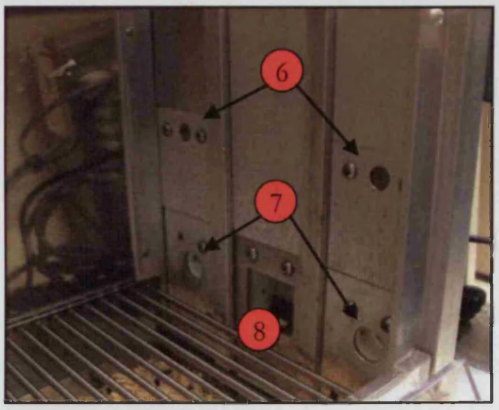


Figure 2.7.4a

1: test chamber. 2: dipper for transferring reinforcer into the food magazine. During a session, it rested within a reinforcer-filled container. 3: chamber floor, either smooth Perspex or a grille (as shown in diagram). 4: house light. 5: loudspeaker. 6: stimulus lights. 7: nose-poke apertures; behind each were a stimulus light and a vertical infra-red beam. 8: food magazine, with a hole to enable the delivery of the reinforcer by the dipper and spanned by an infra-red beam.

2.7.5 Simple visual discrimination and reversal learning task

The simple visual discrimination and reversal learning task utilised a semi-automated 8-arm radial arm maze (Tech[nix], Babraham Institute, U.K.), which had been configured to work as a Y maze (**Figure 2.7.5a**). The Y maze consisted of a transparent Perspex start arm and two opaque Perspex goal arms (black and white), of equal dimensions (345mm × 51mm × 81mm, length × width × height). The arms were removable and slotted into position on the maze. The arms were arranged in a Y shape, extending from a central arena. Infra-red sensors (R.S. components, U.K.) were located on the arms at 20, 70 and 280mm from the centre of the maze; these tracked the activity of the mouse as it moved within the maze, providing information on the subject's arm choice and latencies on various measures. Furthermore, the infra-red sensors were linked to motors (Amerang Ltd., U.K.) that controlled the opening and closing of Perspex guillotine doors around the central arena. At 320mm from the centre of the maze, there was a food well into which the reinforcer (10% condensed milk solution, Nestlé, U.K.) could be placed and the

reinforcer could not be seen from the centre of the maze. The whole maze was elevated at 1m above the ground. The test room was consistently and dimly illuminated with two 60W light bulbs, positioned next to the two choice arms facing away from the maze. An Acorn RISC computer running a programme with custom written software (Arachnid, Cambridge Cognition Ltd., U.K.) was used to operate the maze and record data.

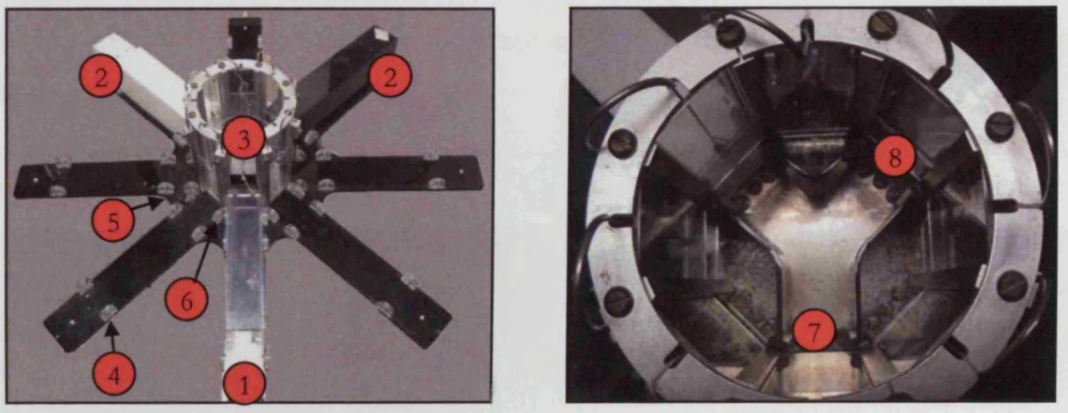


Figure 2.7.5a 1: start arm. 2: two goal arms (one black, one white). 3: central choice arena. 4/5/6: examples of infra-red sensors located in different parts of the maze. 7: Y-shaped choice space. 8: transparent guillotine doors for entry into goal arms.

2.8 Culling protocol

At the end of the experiment (or if the animal was severely unwell) the subjects were culled through cervical dislocation. The femurs of progeny from the XO generating crosses were removed for bone marrow karyotyping. From the Four Core Genotypes animals, the brain and testes were dissected and trunk blood samples were taken.

2.9 Karyotyping and genotyping protocols

2.9.1 Karyotyping XO mice using bone marrow metaphase spreads

Immediately after culling, the femurs were dissected out of the animal and flushed with 0.5ml of RPMI medium (Invitrogen, U.K.) and demecolcine (Sigma-aldrich, U.K.) solution (0.1ml of 0.04% w/v demecolcine stock in 50ml RPMI medium) per leg using a syringe into a 2ml round-bottomed eppendorf tube. The cells were incubated at 31°C for 15 minutes, and then pelleted at 1000g for 5 minutes, resuspended in 2ml 0.56% w/v solution of potassium chloride in water and left at room temperature for 20 minutes. The cells were pelleted again at 1000g for 5 minutes, after which they were washed by fresh fixative (3:1 methanol:glacial acetic acid) five times, with centrifugation in between each wash. After the final wash, the cells suspended in fixative were

dropped onto cleaned slides (cleaned with 2% concentrated hydrochloric acid in 98% absolute ethanol) and stained with 4% Giemsa stain in pH7.4 PBS buffer for 15 minutes. If metaphase chromosomes were not well spread on the slide, cells were washed with fixative and again. Slides were viewed at 100x magnification using an oil immersion lens. The karyotype 39,XO was determined by the presence of 39 chromosomes in four separate spreads. The parental origin of the single X chromosome in the 39,XO animal was indicated from the generating crosses. The karyotype 40,XY*^{X} was determined by the presence of the small Y*^{X} chromosome (see **Figure 2.9.1a**).

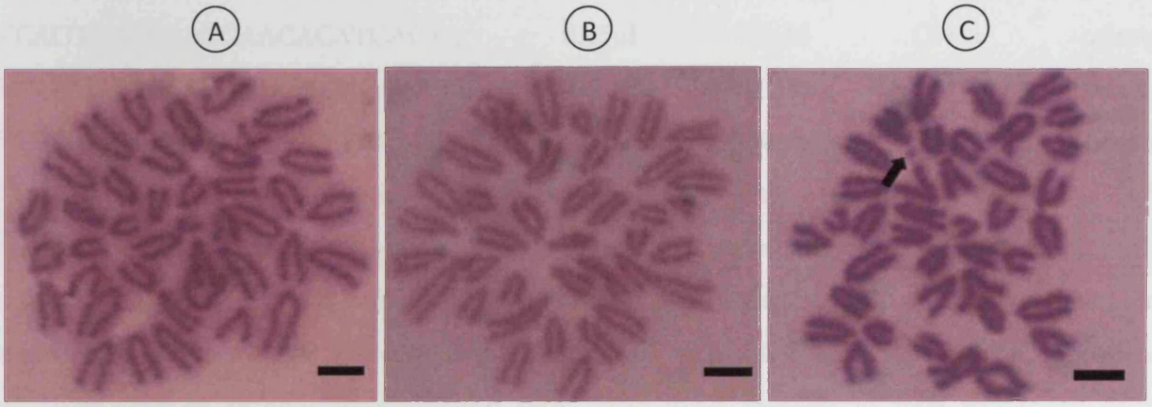


Figure 2.9.1a

A: 40,XX; the chromosome spread clearly showed 40 chromosomes.

B: 39,XO; there were only 39 metaphase chromosomes in this spread.

C: 40,XY*^{X}; the arrow showed the small Y*^{X} chromosome.

Bar = 2μm. Photos courtesy of Dr William Davies.

2.9.2 Genotyping of Four Core Genotypes mice

2.9.2.1 DNA extraction

Tail biopsies from the FCG mice were taken for DNA extraction at weaning, and for purposes of double-checking genotypes, at *post mortem*. The tail sample was incubated with 200μl lysis buffer (100mM Tris-HCL pH8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl) with Proteinase K (0.05mg per ml lysis buffer) at 55°C overnight. The tail lysis mixture was centrifuged at 13000rpm for 10 minutes at 4°C, after which the supernatant was retained. 200μl ice cold isopropanol was added to the supernatant to precipitate the DNA and was left for 30 minutes. The resultant solution was spun at 13000rpm for 10 minutes at 4°C, after which the supernatant was discarded and the DNA pellet left to air dry. The pellet was then resuspended in 20μl TE buffer.

2.9.2.2 Genotyping by polymerase chain reaction (PCR)

The genotypes of the FCG mice could be determined by using a combination of gonadal phenotype information and results from agarose gel electrophoresis of PCR products for the genes *Ssty* and *Myog*. *Ssty* is a Y-linked gene, whilst *Myog* is an autosomal gene that serves as an amplification control.

The following quantities of solutions were added together for the PCR mastermix for a single tail sample; 17.75µl sterile water, 2.5µl 10× buffer, 1µl 5mM dNTPs, 1µl 10µM *Ssty* forward primer (5'CTGGAGCTCTACAGTGATGA3'), 1µl 10µM *Ssty* reverse primer (5'CAGTTACCAATCAACACATCAC3'), 0.25µl 10µM *Om1a* primer (5'TTACGTCCATCGTGGACAGCAT3'), 0.25µl 10µM *Om1b* primer (5'TGGGCTGGGTGTTAGTCTTAT3'), 0.25µl Taq DNA polymerase (HotStarTaq, Qiagen, U.K.). Note that *Om1a* and *Om1b* are primer pairs for the gene *Myog*. 1µl of genomic DNA solution was added to each reaction. The mixture was transferred to a PCR machine (MJ Research Inc., U.S.A.) and subject to the following conditions: 94°C for 15 minutes in initialization step, 94°C for 45 seconds in denaturation step, 61°C for 45 seconds in annealing step, 72°C for 45 seconds in elongation step, 72°C for 5 minutes in final elongation step. Denaturation, annealing and elongation steps were repeated for 35 cycles. PCR products were visualised on a 1.5% ethidium-bromide stained agarose gel using a camera (Hamamatsu, U.S.A.) and UV transilluminator (UVP Inc., U.S.A.) and on a computer running the Labworks software (UVP Inc., U.S.A.).

Gonadal males in which both the *Ssty* and *Myog* genes are amplified must be XY-*Sry*, gonadal males in which only the *Myog* gene is amplified must be XX*Sry*, gonadal females in which both *Ssty* and *Myog* genes are amplified must be XY-, and gonadal female mice in which only the *Myog* gene is amplified must be XX (**Table 2.9.2.2i** and **Figure 2.9.2.2a**).

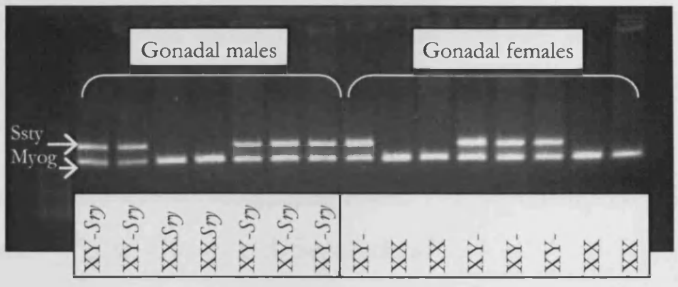


Figure 2.9.2.2a The agarose gel, visualized under UV light, shows 2 distinctive bands, confirming the presence of the genes *Ssty* and *Myog*, with product sizes 343bp and 245bp respectively.

Table 2.9.2.2i shows how each genotype group corresponds uniquely to a combination of external genitalia phenotype and presence of *Ssty* band. The *Myog* band should be present in each sample to demonstrate that DNA amplification has occurred during PCR.

Genotype	External genitalia	Presence of *Ssty* band	Presence of *Myog* band
XX	Female	No	Yes
XY-	Female	Yes	Yes
XX*Sry*	Male	No	Yes
XY-*Sry*	Male	Yes	Yes

2.10 Hormone level determination in blood with ELISA in Four Core Genotypes animals

After the FCG animals were culled via cervical dislocation, trunk blood samples were taken and transferred to blood collection tubes (BD Microtainer tubes, gold; BD, U.S.A.). The microtainer tubes contained a Clot Activator and Gel for serum separation. After the serum had separated, it was transferred to a fresh eppendorf tube and frozen at -20°C.

A testosterone ELISA kit (DRG Instruments GmbH, Germany) was used to assay the level of testosterone in the serum samples. The samples were defrosted immediately prior of assaying. Microtiter wells, coated with a mouse monoclonal anti-Testosterone antibody, were affixed to the microtiter plate reader and 25µl of each serum sample, standard (solutions with testosterone at concentration of 0, 0.2, 0.5, 1.0, 2.0, 6.0 and 16ng/ml, as per provided in the kit) and control (distilled water) solutions were added to the wells. 200µl enzyme conjugate (Testosterone conjugated to horseradish peroxidase) was added to each well and mixed thoroughly, after which the wells were left to incubate for 60 minutes at room temperature. The contents of the well were shaken out and the wells were rinsed three times with 400µl wash solution per well. 200µl substrate solution was added to each well and left to incubate for 15 minutes at room temperature. 100µl stop solution was added to each well and the optical density was read at 450±10 nm with Sunrise, a microplate calibrated reader (Tecan Group Ltd, Switzerland), running on the program XFluor4 (Tecan Group Ltd, Switzerland).

Using SigmaPlot (SYSTAT, U.S.A.), the optical densities of the standard solutions were plotted on a standard curve graph (x axis: testosterone concentration ng/ml; y axis: optical density /

absorbance). With the Regression Wizard function, the standard curve was modelled using the 'Hyperbola 2 decay' model shown below (*a* and *b* are constants to be found).

$$y = \frac{ab}{b+x}$$

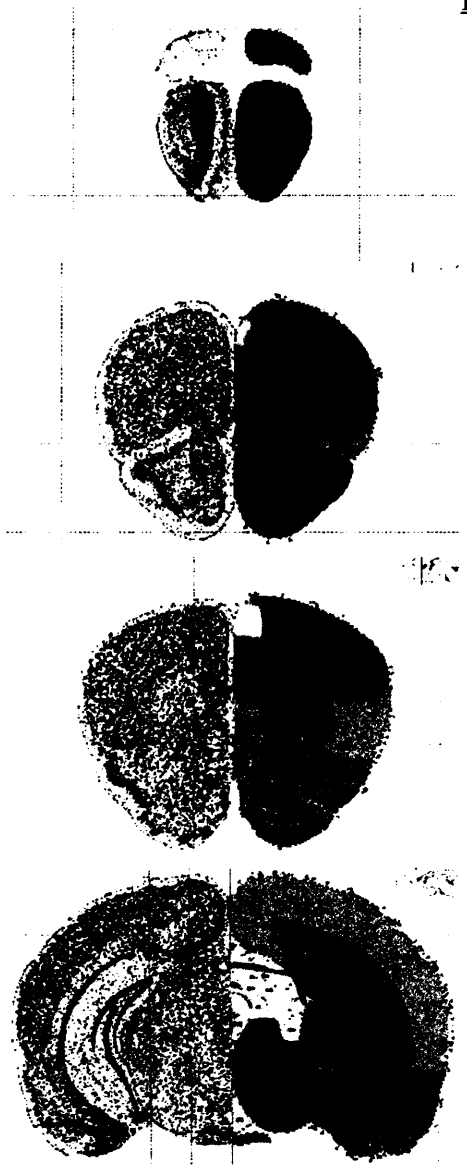
The equation and the optical density readings of serum samples were then used to solve for the sample concentrations of testosterone. The assay dynamic range of the ELISA kit was 0 – 16ng/ml, with minimal cross reactivity with other substances (cross reactivity with testosterone was 100, and highest cross reactivity with other substances was 0.9 with androstenedione). Analytical sensitivity of the assay was 0.083ng/ml.

2.11 Tissue dissection

After behavioural testing, FCG animals were culled via cervical dislocation and their brains were dissected immediately for use in quantitative (real-time) polymerase chain reaction (qPCR) analyses. All brain dissections were performed with a razor blade on a pre-cooled metal plate in order to minimise RNA degradation and allow the tissue to be dissected more easily.

All regions were dissected out from the left hemisphere of the brain (**Figure 2.11a**). The olfactory bulbs were removed by cutting down in front, and underneath, of the frontal cortex, at around Bregma 2.45mm. The frontal cortex was dissected from Bregma 1.045mm, at an angle down to Bregma 3.245mm to the front of the brain. The angle of cutting avoided the underlying striatum, and the dissection of the frontal cortex contained prelimbic, infralimbic, cingulate and orbitofrontal cortices, together with overlying motor and premotor cortex. The midbrain was dissected from a coronal slice of 1mm, at Bregma -3.28mm to -4.28mm, and included the regions of ventral tegmental area and substantia nigra. Testes were dissected out of the scrotum using forceps and razor blades, and weighed immediately. Dissected brain regions and testes were frozen rapidly in dry ice, and stored at -80°C. After each dissection, the razor blade was discarded and all equipment was cleaned with 75% ethanol to prevent RNA contamination.

Figure 2.11a



Coronal slices from Bregma (mm):

+3.245

+2.245

+1.545

-3.28

Key for brain dissection:

The frontal cortex was cut, at an angle, from 1.045mm, down to 3.245mm. The dissected tissue comprised of the following regions, as shown on the left:

- Motor and premotor cortex
- Anterior cingulate
- Prelimbic cortex
- Orbitofrontal cortex
- Infralimbic cortex
- Olfactory bulb (removed)

The midbrain section was dissected out from a 1mm coronal slice at Bregma -3.28mm. The area taken included the midbrain, ventral tegmental area and substantial nigra, as indicated on the left:

- Midbrain reticular nucleus
- Ventral tegmental area
- Substantial nigra

2.12 Additional general control measures

Care was taken to minimise sources of experimental noise. In many cases, where practicable, the researcher was blind to factor genotype or karyotype. For the behavioural work, testing was done at the same time of day (between 0700 and 1900 hours) in order to minimize circadian effects and other factors were kept constant, such as access to water following testing and general testing room conditions. The running order of subjects in experiments was pseudorandomised to minimise order effects. To reduce cage and litter effects, all animals were housed in groups and subjects were taken from as many cages as possible. In maze-based tasks where subjects were tested sequentially one after another, males were tested before females in

order to minimise disruption to the performance of males due to female scent that might remain despite cleaning of the apparatus after every trial. At the beginning of each behavioural test session, subjects were taken to the test room in their home cages and allowed to habituate to the testing room environment for at least 15 minutes. The testing room was air-conditioned and its temperature was maintained at around 22°C, with approximately 50% humidity. The room was lit by fluorescent ceiling lights, 60 watts desk lamps or a 40 watts red light, depending on the behavioural test being carried out at the time. Overall light intensity ranged from 5-10 lux. Computer and electronic equipment provided constant background noise. The room was cleaned thoroughly once a week.

For the *post-mortem* analyses (blood hormone determinations, brain and testis dissections) procedures were carried out in a single batch at the same time of day. In all cases, animals were placed back on free home cage water for at least one week prior to culling.

A number of controls were included in the experiments using the mouse lines to account for more specific litter, maternal and cross effects. These are described in detail in the relevant experimental chapters.

2.13 Data presentation and statistical methods

All quantitative data were presented as mean values +/- standard error of the mean (SEM) unless stated otherwise. All statistics were analysed using SPSS (version 17, SPSS Inc., IBM, U.S.A.). Data were analysed by ANOVA (if normally distributed as indicated by Levene's test) or where appropriate, Repeated Measures ANOVA. For data not normally distributed, a relevant Kruskal-Wallis Test was used; in the case of a Repeated Measure design, data not normally distributed was analysed using Friedman Test. Where significant main effects and interactions were identified in the main ANOVA, *post hoc* testing (Tukey HSD and Least Significant Difference tests, respectively) permitting specific pair-wise comparisons was carried out (see individual experimental chapters for details). Huynh-Feldt and Greenhouse-Geisser corrections were applied as necessary in Repeated Measures ANOVA, and adjusted degrees of freedom are provided. Chi-square test for goodness of fit was carried out on non-parametric data. For all comparisons, p values of <0.05 were regarded as significant. See individual experimental chapters for specific factors used in the statistical comparisons of data and for non-standard statistical design.

Chapter III

Initial physiological and behavioural phenotyping of the XO mouse model

3.1 Introduction

The experiments in this chapter were concerned with (i) establishing the basic physical and behavioural competence of the XO mouse model and (ii) recognising a number of potential confounds in the interpretation of data obtained with this model stemming from the way in which the 39,$X^{m}O$ and 39,$X^{p}O$ mice were created. Although there have been published reports of the use of the XO model in behavioural studies (Davies *et al.*, 2005a; Isles *et al.*, 2004), it was still important to establish their general competency before moving on to the more complex, and time-consuming, behavioural assays described later in the thesis. This was important not just in terms of whether the mice could perform the tasks in the first place but also in terms of potential confounds in the interpretation of the behavioural data obtained from the more complex behavioural and cognitive assays. For example, an apparent effect on learning may be due to more fundamental and simple (though subtle) effects on sensory, motor or motivational processes. Also, previous reports had covered work done in Cambridge, whereas this work was carried out in Cardiff with different researchers and a completely new generation of mice.

The behavioural battery of tests used in this experimental chapter included physiological evaluation of body weight and oestrous cycle, and behavioural assessment of subjects' performance on the rotarod, in locomotor activity boxes and on the elevated zero maze. Briefly, the rotarod apparatus tests the subject's motoric competence and stamina by looking at its ability to stay on a rotating rod (Jones & Roberts, 1968; Crawley *et al.*, 1999; Fujimoto *et al.*, 2004), while the locomotor activity box measures animals' general movement and locomotor behaviour (Wahlsten *et al.*, 2003). The elevated zero maze allows subjects to freely explore a circular platform, which consists of 'closed' areas that have high walls on either side and of 'open' areas without the walls. By examining the frequency and duration in which animals venture into the more anxiogenic 'open' areas, one can assess the general fear reactivity of the subject (Shepherd

et al., 1994; Cook *et al.*, 2001; Cook *et al.*, 2002). These behavioural tests are well established in my laboratory (Davies, 2003, Ph.D thesis; Relkovic, 2009, Ph.D thesis).

As introduced in the General Introduction, the XO mouse model allows dissociations between effects due to the parental origin of the single X chromosome and X-monosomy effects. Differences between 39,$X^{m}O$ and 39,$X^{p}O$ animals could point to a genuine X-linked parent-of-origin effect. However, it is important to be aware of the fact that the 39,$X^{m}O$ and 39,$X^{p}O$ animals were generated by two separate crosses with different mothers and littermates (specific details of the XO generating crosses are described in the General Introduction, and summarised again in Section **3.2** below). Whilst it was unlikely, *a priori*, that these general factors could have impacted in any major way on the data obtained in the XO mice, it was considered necessary to control for the possibility by testing 40,XX^{Paf} and 40,$X^{Paf}X$ animals (i.e. the resulting female littermates with the normal XX karyotype of the two generating crosses) alongside the 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX animals. If a difference in behaviour between 39,$X^{m}O$ and 39,$X^{p}O$ animals (possibly contributed by X-linked parent-of-origin effects) coincides with a difference between females from the 39,$X^{m}O$-generating cross (40,XX^{Paf}) and females from the 39,$X^{p}O$-generating cross (40,XX and 40,$X^{Paf}X$), this may suggest that the possible X-linked parent-of-origin effect might have been confounded by maternal and littermate influences. This 'cross' confound does not apply to where X-monosomy effects are detected, since in that case, effects are common to both 39,$X^{m}O$ and 39,$X^{p}O$ animals. Therefore, the data from the 40,XX^{Paf}, 40,$X^{Paf}X$ comparisons (the *Paf* comparison) were only presented in the Results section when there was an apparent parent-of-origin effect in the XO comparison. Given that the animals from the XO mouse model have displayed no gross morphological abnormalities (Lynn & Davies, 2009) and no significant differences were found in motoric competence and reactivity to novelty (Davies, Ph.D thesis, 2003), I would not expect to find differences between the XO mice in the rotarod and the elevated zero maze tasks. However, X-monosomy and X-linked imprinting effects in fear reactivity have been observed in the elevated plus maze (Davies, Ph.D thesis, 2003; Isles *et al.*, 2004), and therefore I would expect to see behavioural differences in the XO mouse model on the elevated zero maze.

3.2 Materials and methods

3.2.1 Generation of XO mice

The XO mice used in this and subsequent experimental chapters were generated using two separate crosses. Full details of the cross particulars are described in the General Introduction. **Figure 3.2.1a** presents a simple overview of the two crosses I used. Briefly, in the generation of 39,$X^{m}O$ animals, there is a possibility of chromosome loss in male gametes during chromatid recombination in male gametogenesis; this possibility is increased by the presence of the *Paf* mutation. Fertilisation of a normal female egg with a sex chromosome-null male sperm will result in 39,$X^{m}O$ female offspring. In the generation of 39,$X^{P}O$ animals, the large paracentric chromosomal inversion In(X)1H in the mother promotes crossing over between chromosomes during gametogenesis and chromosome loss in female gametes may occur. Fertilisation of the sex chromosome-null female gamete with a normal male gamete will result in 39,$X^{P}O$ female offspring. Note that the *Paf* mutation is not necessary in the production of 39,$X^{P}O$ animals; this mutation is introduced in the mother so that both 39,$X^{m}O$- and 39,$X^{P}O$- generating crosses will produce female littermates with a *Paf* mutation (40,XX^{Paf} and 40,$X^{Paf}X$ animals, respectively). As explained in the General Introduction, whilst this serves to help reduce gross littermate effects, this strategy does not remove confounds completely as parent-of-origin *Paf* effects may still be present.

Figure 3.2.1a

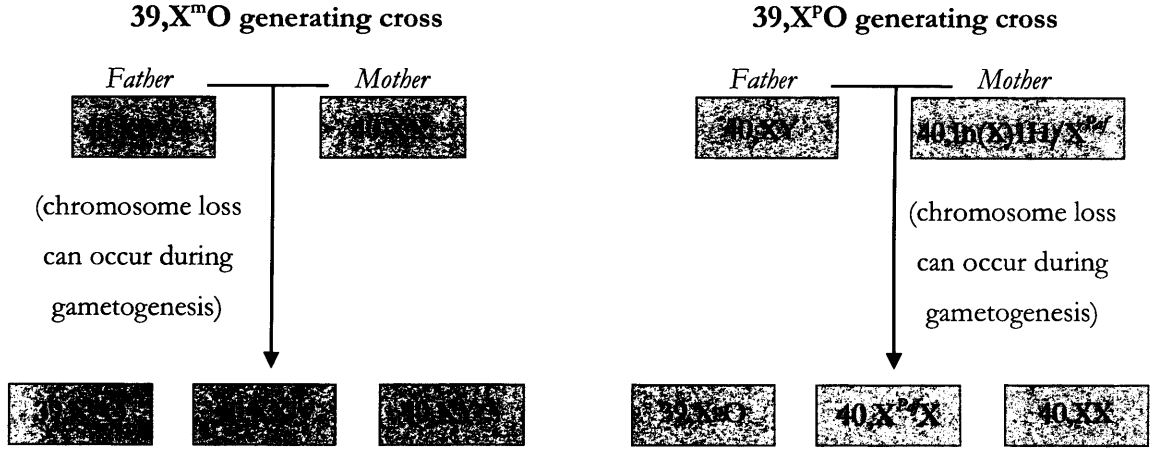


Figure 3.2.1a Breeding strategies for generating 39,$X^{m}O$ and 39,$X^{P}O$ animals. The diagram only includes the female offspring generated and used in this thesis.

3.2.2 Subject numbers and animal husbandry

In this experimental chapter, animals from the XO mouse model (39,$X^{m}O$, 39,$X^{P}O$ and 40,XX) and associated female littermates (40,XX^{Paf} and 40,$X^{Paf}X$) were subject to various physiological and behavioural tasks. Animals were aged from six to 12 months old. **Table 3.2.2i** shows the number of subjects tested in the various tasks used in this chapter. General housing, handling and behavioural testing conditions were as described in Chapter II, **2.2** and **2.3**.

Table 3.2.2i Numbers of subjects (n) tested in the various physiological and behavioural tasks.

Test	Karyotype and 'n'				
	39,$X^{m}O$	39,$X^{P}O$	40,XX	40,XX^{Paf}	40,$X^{Paf}X$
Body weight	15	12	9	15	10
Oestrus cycle length	14	12	9	15	9
Rotarod	15	12	9	14	10
Locomotor activity	14	11	8	11	10
Elevated zero maze	15	12	9	12	10

3.2.3 Physiological assessments

3.2.3.1 Body weight measurements

As the mice were born and housed initially at MRC, NIMR (Mill Hill, London), it was not possible to determine the developmental weight measurements. For this study, stable body weights were determined when animals were five months old, and again at 11 months old. Animals had ample time for habituation to housing environment and were accustomed to daily handling. Animals were weighed at the same time every day for five days. *Ad libitum* access to food and water was available.

3.2.3.2 Oestrous cycle

Vaginal smearing was performed as described in Chapter II, **2.4**. The data presented in this chapter was from an initial assessment in mice aged six months.

3.2.4 Behavioural assessments

3.2.4.1 Rotarod

Subjects were tested on the rotarod apparatus (described in Chapter II, **2.7.1**) to test their motor function and balance under conditions of accelerating and constant rotation speed. On day One of testing (accelerating speed block), animals received a total of five trials, each of five minutes duration, on the rotarod, with speed accelerating uniformly from 5 to 50 rpm in the five minute trial. The first three trials were given consecutively, and if an animal fell off the rotarod during the first ten seconds, it was put back on immediately. Following these initial trials there was an interval of two hours to ensure that the animals did not suffer from fatigue, after which a further two 'probe' trials were administered. The speeds at which a subject fell off the apparatus during the two probe trials were recorded. On day Two of testing (constant speed block), animals were given a total of eight trials; two trials for each of the four constant rotation speeds, 15, 25, 35 and 45 rpm. Each trial lasted a maximum of 60 seconds, and the latency to fall off the apparatus was recorded.

3.2.4.2 Locomotor activity

Movements in, exploration of, and habituation to a novel environment by subjects were tested in the locomotor activity boxes, described in Chapter II, **2.7.2**. For three consecutive days, subjects were put into the clear Perspex boxes for two hours daily. Animals were run in the dark at the same time of day. The number of infra-red beam breaks and the number of runs (when both beams located at either end of the activity box were broken consecutively, which signified the animal running across the cage) were recorded by a computer every five minutes for two hours every day (over 24 bins) with custom written BBC BASIC V6 programmes on the ARACHNID system (Cambridge Cognition Ltd., Cambridge, U.K.). The distinction between beam breaks and runs revealed different types of motor behaviour, beam breaks indexing general movement and runs, where the animal had to make successive beam breaks at either end of the testing box, indexing more global locomotor activity. The boxes were thoroughly cleaned with 1% acetic acid after each animal. Animals were tested for three consecutive days to access habituation to the novel environment.

3.2.4.3 Elevated zero maze

Fear reactivity was assessed using the elevated zero maze, described in Chapter II, **2.7.3**. Animals were transferred in their home cages to the testing room, lit with a 40W red light bulb,

15 minutes before start of testing, so that animals could habituate to the testing environment. All animals started the trial in the right closed quadrant on the zero maze. The animals were then allowed to explore the maze freely for five minutes, after which the maze was cleaned thoroughly with 1% acetic acid. If an animal fell off the maze, it was immediately put back on the apparatus. The subjects were tracked using the Ethovision tracking system (Noldus, U.K.); the maze arena was separated into four zones: two open zones (top and bottom) and two closed zones (left and right). The closed zones were designated to be hidden because the high walls prevented the detection of the albino animals. The main measures indexing fear reactivity were time spent in the open parts of the maze and entries into the open areas.

3.2.5 Statistical analyses and additional control measures

Statistical data were analysed using SPSS software (version 17, SPSS Inc., IBM, U.S.A.). I performed two comparisons, each of which was subject to One Way ANOVA. As noted in the introduction section to this chapter, Comparison 2 was only performed where an X-linked parent-of-origin effect was apparent in Comparison 1. The *Paf* control data from Comparison 2 were included in the results in this case.

Comparison 1: 'XO comparison' (Between Subjects factor KARYOTYPE): 39,$X^{m}O$, 39,$X^{p}O$, 40,XX. The purpose of this main comparison was to check for effects of X-monosomy and X-linked parent-of-origin effects.

Comparison 2: '*Paf* comparison' (Between Subjects factor GENOTYPE): 40,XX, 40,XX^{Paf}, 40,$X^{Paf}X$. An additional comparison was made between females from the 39,$X^{m}O$-generating cross (40,XX^{Paf}) and females from the 39,$X^{p}O$-generating cross (40,XX and 40,$X^{Paf}X$). The purpose of this second comparison was to check for gross cross effects only where differences between 39,$X^{m}O$ and 39,$X^{p}O$ mice were found.

Repeated Measures ANOVA was used when appropriate; additional factors specific to the analysis were defined in the relevant Results section, and furthermore, Greenhouse-Geisser (epsilon of 0.75 or lower) or Huynh-Feldt (epsilon of 0.75 or higher) corrections were applied to degrees of freedom if the Mauchly's Test of Sphericity was violated in Repeated Measure tests. When initial ANOVA revealed a significant effect, Tukey HSD Test was performed for *post hoc* comparisons. For all comparisons, p values of <0.05 were regarded as significant.

3.3 Results

3.3.1 Physiological data

3.3.1.1 Body weight

Average body weight measured over five days from 39,X^mO, 39,X^pO, 40,XX in animals aged five months did not differ significantly (**Figure 3.3.1.1a**; effect of KARYOTYPE, $F_{2,33} = 2.851$, n.s.). Later on at 11 months old there were still no weight differences between 39,X^mO, 39,X^pO, 40,XX animals (**Figure 3.3.1.1b**; effect of KARYOTYPE, $F_{2,33} = 2.333$, n.s.).

Figure 3.3.1.1a

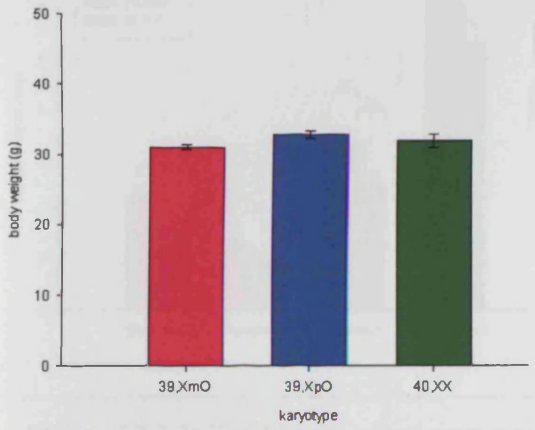


Figure 3.3.1.1b

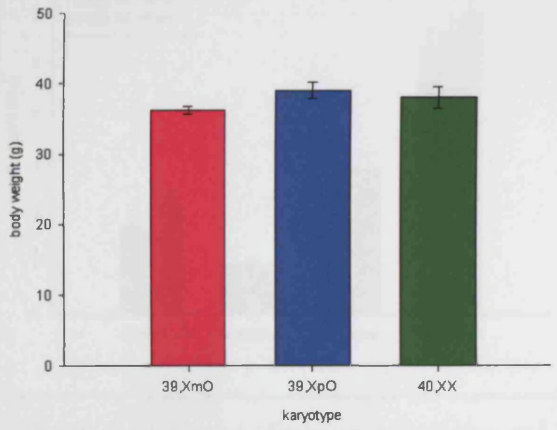


Figure 3.3.1.1a The baseline body weight of the XO comparison at five months old, with *ad libitum* food and water. Data are presented as mean values with ± SEM.

Figure 3.3.1.1b The baseline body weight of the XO comparison at 11 months old, with *ad libitum* food and water. Data are presented as mean values with ± SEM.

3.3.1.2 Oestrous cycle

Repeated Measures ANOVA, with the Within Subject factor STAGE (diestrous, proestrous and oestrous), revealed no significant differences in the cycle length between 39,X^mO, 39,X^pO and 40,XX animals generally across the three cycle stages (**Figure 3.3.1.2a**; effect of KARYOTYPE, $F_{2,32} = 1.9$, n.s.). However, there was a STAGE × KARYOTYPE interaction ($F_{3.025,4.8399} = 2.182$, $p<0.02$), and *post hoc* comparisons revealed a difference between 39,X^mO and 39,X^pO subjects in diestrous (longer in 39,X^mO) and oestrous (longer in 39,X^pO) stages, and a difference between 40,XX and 39,X^pO females in oestrous (longer in 39,X^pO) stage. There was also a significant general difference in the cycle lengths between diestrous, proestrous and oestrous stages (effect

of STAGE, $F_{1.512,48.399}$ = 52.744, p<0.001), with proestrous being shorter than diestrous and oestrous stages across all karyotype groups.

In the *Paf* comparison (**Figure 3.3.1.2b**) there was no difference between 40,XX, 40,XX^{Paf} and 40,X^{Paf}X in cycle length (effect of GENOTYPE, $F_{2,30}$ = 6.670, n.s.) and, unlike the XO comparison above, no STAGE × GENOTYPE interactions ($F_{4,60}$ = 1.183, n.s.). As before, there were general effects of stage of oestrous, with proestrous shorter than diestrous and oestrous stages (effect of STAGE, $F_{2,60}$ = 59.761, p<0.001).

Figure 3.3.1.2a

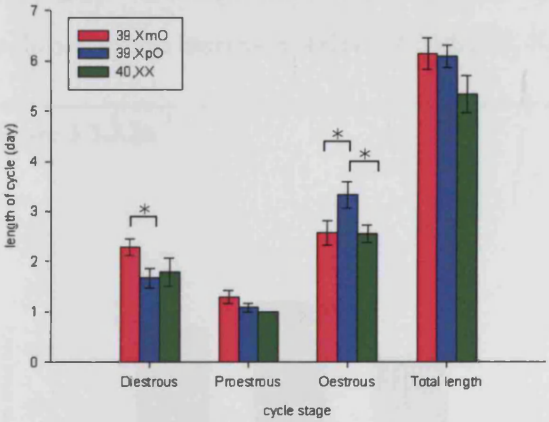


Figure 3.3.1.2b

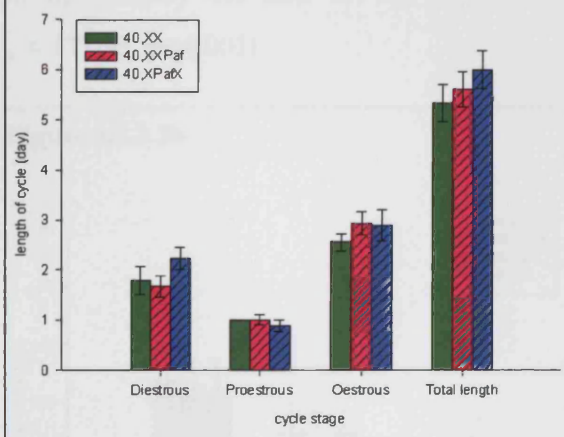


Figure 3.3.1.2a Total length of oestrous cycle and length at each stage (diestrous, proestrous and oestrous) in the XO comparison as determined by vaginal smears from female mice aged six months. Data are presented as mean values with ± SEM. * p<0.05

Figure 3.3.1.2b Total length of oestrous cycle and length at each stage (diestrous, proestrous and oestrous) in the *Paf* comparison as determined by vaginal smears from female mice aged six months. Data are presented as mean values with ± SEM.

3.3.2 Behavioural data

3.3.2.1 Reactivity to handling

During the initial handling of the subjects, all animals showed mild signs of distress, which included urination and fecal deposition. After three days of handling, these signs had subsided in most animals. There did not appear to be an obvious effect of karyotype or the *Paf* mutation, on the degree of initial distress and habituation. Vaginal smearing led to some signs of distress in females, including urination and faecal deposition, but again, most signs subsided after three days of habituation to the procedure.

indexed by infra-red beam breaks obtained over three days of testing. 39,X^{m}O, 39,X^{p}O and 40,XX animals did not vary in activity (**Figure 3.3.2.3a**; effect of KARYOTYPE, $F_{2,30} = 1.624$, n.s.), and showed significant decrease in activity across DAY, indicating habituation to the novel testing environment ($F_{1.775,53.264} = 13.402$, $p<0.001$); *post hoc* tests revealed significant differences between Day One and Two, and between Day One and Three. No interaction between DAY and KARYOTYPE was noted ($F_{3.551,53,264} = 0.45$, n.s.). In concordance with the infra-red beam breaks data, activity data indexed by number of runs (**Figure 3.3.2.3b**) also showed that 39,X^{m}O, 39,X^{p}O and 40,XX did not vary in activity (effect of KARYOTYPE, $F_{2,30} = 2.123$, n.s.). Activity decreased significantly over DAY as animals habituated to the environment ($F_{1.811,54.342} = 4.911$, $p<0.02$), with a significant difference between DAY One and Three as revealed by *post hoc* tests. No interaction between DAY and KARYOTYPE was noted ($F_{3.623,54.342} = 0.916$, n.s.).

Figure 3.3.2.3a

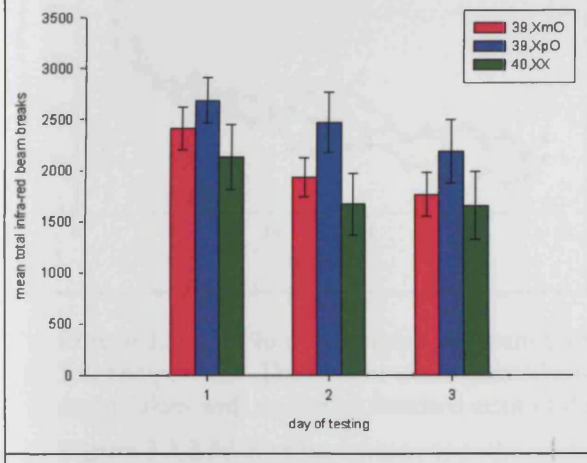


Figure 3.3.2.3b

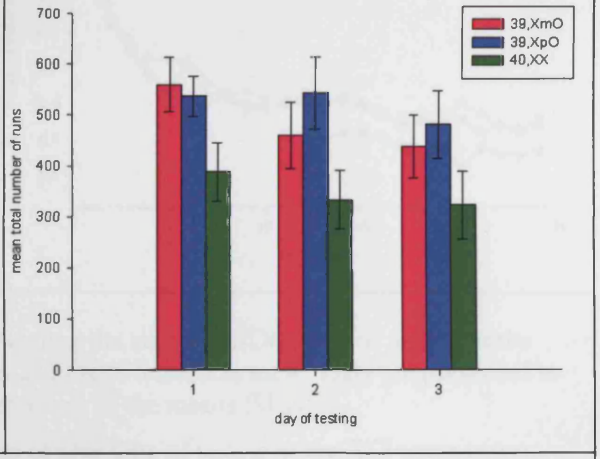


Figure 3.3.2.3a Locomotor activity, as indexed by the number of infra-red beam breaks, over three consecutive days of testing in the XO comparison. Data are presented as mean values ± SEM.

Figure 3.3.2.3b Locomotor activity, as indexed by the number of runs, over three consecutive days of testing in the XO comparison. Data are presented as mean values ± SEM.

(ii) Five-minute bin data over Day One:

The number of infra-red beam breaks and runs on the session on Day One of testing, over 24 bins of five minutes each, were analysed by Repeated Measures ANOVA with Within Subject factor of BIN (i.e. Bin 1-24). Only Day One was analysed as I would like to look at the *initial* habituation behaviour of subjects within a session. As the session proceeded, 39,X^{m}O, 39,X^{p}O and 40,XX animals showed significantly reduced activity as indexed by beam breaks (**Figure**

3.3.2.3c; effect of BIN, $F_{6.4,192.006} = 52.598$, $p<0.001$), which was expected as subjects habituated to the novel environment over time. Activity over the session did not differ between subject groups (effect of KARYOTYPE, $F_{2,30} = 1.121$, n.s.), and there was no interaction between BIN and KARYOTYPE ($F_{12.8,192.006} = 1.598$, n.s.). With regard to activity as indexed by runs (**Figure 3.3.2.3d**), 39,X^{m}O, 39,X^{p}O and 40,XX again showed significantly decreased activity over the session (effect of BIN, $F_{5.988,179.638} = 51.150$, $p<0.001$), but there was no difference between groups (effect of KARYOTYPE, $F_{2,30} = 2.789$, n.s.). There was also no interaction between BIN and KARYOTYPE ($F_{11.976,179.638} = 1.086$, n.s.) in the run data.

Figure 3.3.2.3c

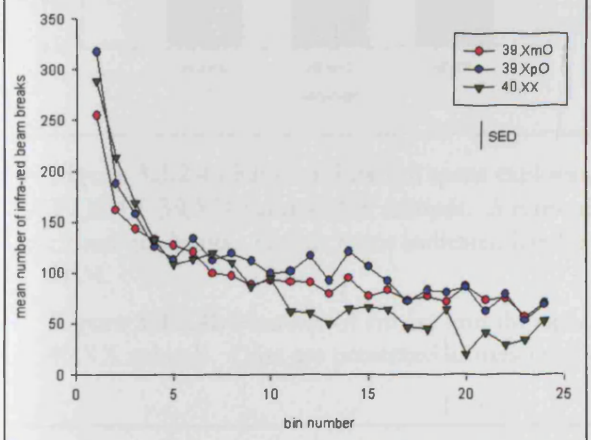


Figure 3.3.2.3d

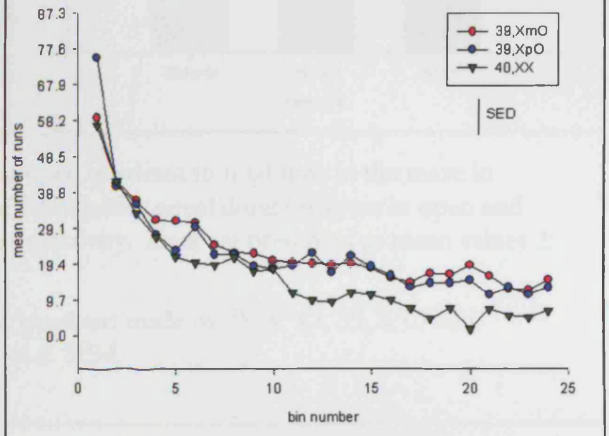


Figure 3.3.2.3c Number of infra-red beam breaks over the session of Day One of testing in the XO comparison. The session was separated into 24 bins of 5 minutes each. Data are presented as mean values with an overall standard error of difference of the means (SED).

Figure 3.3.2.3d Number of runs over the session of Day One of testing in the XO comparison. The session was separated into 24 bins of 5 minutes each. Data are presented as mean values with an overall standard error of difference of the means (SED).

3.3.2.4 Elevated zero maze

The duration in the open and enclosed parts of the elevated zero maze was calculated as ratios of open quadrant duration to total duration on the maze. The means and SEM of the durations spent in open quadrants of the maze were 24.96s±7.17 for 39,X^{m}O, 15.00s±3.51 for 39,X^{p}O, and 10.04s±2.02 for 40,XX animals. All animals spent most of the trial exploring the closed rather than open quadrants of the maze, which suggests that the animals did find the open quadrants to be anxiety-inducing (as shown in **Figure 3.3.2.4a**, with ratios lower than 0.5). 39,X^{m}O, 39,X^{p}O and 40,XX did not vary significantly in the ratio of open quadrant duration to

total duration (**Figure 3.3.2.4a**; effect of KARYOTYPE, $F_{2,33} = 1.050$, n.s.), nor in number of open quadrant entries (**Figure 3.3.2.4b**; effect of KARYOTYPE, $F_{2,33} = 0.482$, n.s.).

Figure 3.3.2.4a

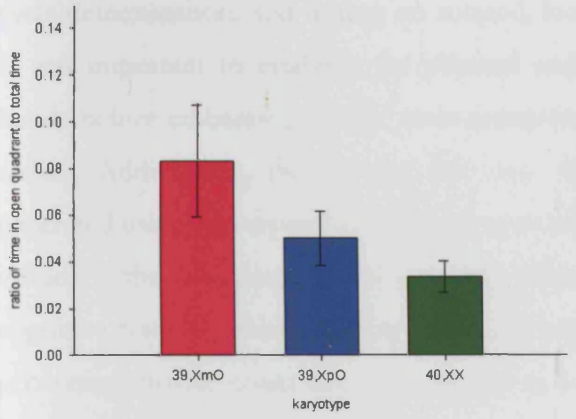


Figure 3.3.2.4b

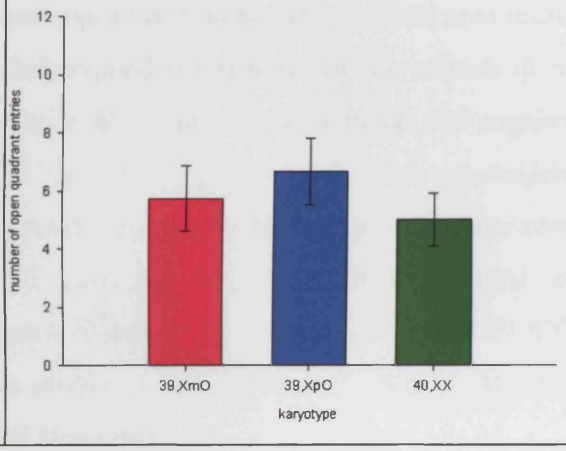


Figure 3.3.2.4a Ratio of duration spent exploring open quadrant to total time in the maze in 39,X^{m}O, 39,X^{p}O and 40,XX animals. A ratio of 0.5 signifies equal duration spent in open and closed quadrants. Higher ratios indicated less fear reactivity. Data are presented as mean values ± SEM.

Figure 3.3.2.4b Number of entries into the open quadrant made by 39,X^{m}O, 39,X^{p}O and 40,XX animals. Data are presented as mean values ± SEM.

3.4 Discussion

This chapter was concerned with the basic physiological and behavioural characterisation of the animals from the XO mouse model (39,$X^{m}O$, 39,$X^{p}O$ and 40,XX; along with 40,XX^{Paf} and 40,$X^{Paf}X$), using a standard, well established battery of tasks(body weight measurements, oestrous cycle determination, and testing on rotarod, locomotive activity boxes and elevated zero maze). It was important to establish the physical and behavioural competence of the animals in my hands before embarking on the more complex and time consuming behavioural and cognitive tasks. Additionally, due to the fact that 39,$X^{m}O$ and 39,$X^{p}O$ animals were unavoidably generated using two separate crosses, it was necessary to note any potential confounds that could influence the interpretation of any data obtained from the more complex behavioural and cognitive tests in subsequent experimental chapters. Litter sizes of the 39,$X^{m}O$- and 39,$X^{p}O$-generating crosses could not be examined as the animals were bred at MRC, NIMR (Mill Hill, London) rather than in the animal house at Cardiff University.

There was a X-linked parent-of-origin effect on oestrus stage; specifically, 39,$X^{p}O$ showed a longer oestrous stage length than 39,$X^{m}O$ and 40,XX animals, and 39,$X^{p}O$ also showed a longer diestrous stage length than 39,$X^{m}O$ animals. This parent-of-origin effect was unlikely to be confounded by cross effects as there were no differences between 40,XX, 40,XX^{Paf} and 40,$X^{Paf}X$ animals. These differences in oestrous cycle lengths might be contributed by hormonal variations (Hawkins & Matzuk, 2008) as well as genetic influences, as demonstrated in a study comparing cycle lengths in different inbred mouse strains (Nelson *et al.*, 1992). An X-linked imprinted gene in sheep has been found to affect ovulation rate (Davis *et al.*, 2001), which is interesting in the light of the parent-of-origin effect on cycle length found in this chapter. In humans, Turner Syndrome (TS) is characterised by infertility and hypogonadism, but there has been no known research into altered cycling and parental origin of the single X chromosome in TS girls. The fertility problems present in human TS girls are absent in the XO mouse model (even though there was a difference in cycle lengths between the animals, 39,$X^{m}O$ and 39,$X^{p}O$ animals have not stopped cycling altogether and are fertile) which might be due to differences in the degree of X chromosome inactivation between human and mouse; around 15% of the human X-linked genes escape X inactivation(Heard & Disteche, 2006), whereas the mouse X chromosome is more extensively inactivated with fewer genes escaping X inactivation, so that in humans, X-monosomy poses to be a greater problem than in mice. It would be interesting to measure pituitary hormones (follicle stimulating hormone and luteinizing hormone) and gonadal hormones such as oestrogen and progesterone in 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX animals to

investigate whether the cycle length variations were due to differences in hormone levels, or due to the parental origin of the X chromosome.

There were no body weight differences between 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX animals both at five months or at 11 months. Burgoyne and colleagues (2002) have previously shown an initial growth deficit in both XO groups, which could not be corrected by introducing a Y^{*X} chromosome. However, adding a Y chromosome lacking *Sry* removed the deficit, which suggests that the weight deficit observed was due to one (or more) X-linked gene which escapes X inactivation and has a functional Y homologue. This reported weight deficit in XO animals appears to disappear by age of five months, as shown in this chapter.

There were no gross group effects in motoric competency as indexed by performance on the rotarod, and no effects in exploratory behaviour and general movement as shown in their locomotive activity. No group differences were found in anxiety as indexed by animals' behaviour on the elevated zero maze, suggesting that there were no significant confounds which could affect the interpretation of data from subsequent cognitive assays. The lack of behavioural differences on the elevated zero maze somewhat conflicts a previous study reporting increased anxiety on the elevated plus maze in both 39,$X^{m}O$ and 39,$X^{p}O$, when compared to 40,XX, animals (Isles *et al.*, 2004). The discrepancy might be due to the use of different apparatus; while the study by Isles *et al.* utilised the plus maze, the present study used the elevated zero maze. The plus maze contains a central area and dead ends for the open and closed arms, whereas the zero maze provides uninterrupted exploration and removes the more 'ambiguous' central area (as it is debatable what sort of behaviour is indexed by time spent in the central area), arguably providing a more sensitive tool to measure anxiety-related behaviours (Shepherd *et al.*, 1994; Cook *et al.*, 2002). There was a difference in age between the animals tested; subjects were aged 12 months in the current study whereas subjects were aged 12 weeks in the Isles study. General experimental set up could contribute to the discrepancy in the results as the Isles study was run in low level white and red light, and the present study was run exclusively in red light. There is also the possibility of floor effects in the results obtained from the present experiment; animals across all karyotype groups did not spend a lot of time in the open quadrants at all (ratios of time spent in open to closed quadrants were much lower than 0.5) and so any potential group effects might not appear. One might consider repeating the elevated zero maze with a lower level of background noise and longer period of habituation in the testing room to reduce anxiety, so that animals might be more inclined to explore the maze and hence, reducing potential floor effects. Other behaviours within the maze, such as rearing and stretch attend, could be further useful

indicators of anxiety; for example, stretch attends reflect 'risk assessment' behaviour and the animal's reluctance to move from the present position for exploration, and a high number of such behaviour would suggest higher levels of anxiety (Blanchard *et al.*, 2001; Walf & Frye, 2007). It was problematical to monitor such behaviours in the current study, as the closed quadrants were flanked by high walls, making it impossible for Ethovision to track the behaviour of subjects within closed quadrants and it was difficult for the researcher to monitor behaviour without standing too close to the maze and potentially affecting the animals' behaviour on the maze.

Work detailed in this experimental chapter has established the general competency of animals from the XO mouse model, allowing further, more complex, cognitive and behavioural assays. There were no gross group effects found in a wide range of physiological and behavioural tasks.

3.5 Summary

- There were no significant body weight differences at both five months and 11 months of age between 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX animals.
- There were subtle parent-of-origin effects on oestrous stage length, with 39,$X^{p}O$ having a longer oestrous stage length than 39,$X^{m}O$ and 40,XX animals. 39,$X^{p}O$ also had a longer diestrous stage length than 39,$X^{m}O$ animals.
- There were no significant differences in the performance on the rotarod between 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX animals.
- There were no significant differences in locomotor activity between 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX animals.
- There were no significant differences in the behaviour on the elevated zero maze between 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX animals.

Chapter IV

Biconditional discrimination and response conflict in XO mouse model

4.1 Introduction

This experimental chapter examined the possibility of X-monosomy and/or X-linked parent-of-origin effects on biconditional discrimination learning and response conflict with the use of the XO mouse model. Previous work with the XO mouse model had shown an X-linked parent-of-origin effect in reversal learning (in both behavioural perseveration and acquisition of new stimulus-reinforcer contingencies), whereby 39,$X^{m}O$ female mice committed more perseverative and reacquisition errors than 39,$X^{p}O$ and 40,XX animals following reversal (Davies, Ph.D thesis, 2003; Davies *et al.*, 2005a). This increased perseverative responding in 39,$X^{m}O$ mice could be mediated by effects of one or more X-linked imprinted genes, with *Xlr3b* being a strong candidate gene, in the brain (Davies *et al.*, 2005a; Raefski & O'Neill, 2005). Neural substrates that have been shown to underlie reversal learning include orbitofrontal cortex (Dias *et al.*, 1996), mediodorsal nucleus of the thalamus (Chudasama *et al.*, 2001) and hippocampus (Murray & Ridley, 1999). Additionally, there is some preliminary evidence that tissue dopamine levels are lower in the medial prefrontal cortex of 39,XO mice than of 40,XX animals, with dopamine levels being higher in the orbitofrontal cortex of 39,$X^{m}O$ mice than of either 39,$X^{p}O$ or 40,XX female mice (Davies, Ph.D thesis, 2003). The above findings using the XO mouse model recapitulated the parent-of-origin effects in behavioural inflexibility and social cognition in Turner Syndrome (TS); Skuse and colleagues (1997) have shown that, using a same-opposite word task, that 45,$X^{m}O$ females performed worse than 45,$X^{p}O$ and 46,XX females, and this impairment might mediate the poorer social cognition observed in 45,$X^{m}O$ subjects.

Since the XO mice appear to display some cognitive deficits also seen in TS females, it was of interest to examine whether other TS cognitive impairments might be observed in the XO mouse. In addition to problems in social cognition (Skuse *et al.*, 1997) and behavioural inhibition (Romans *et al.*, 1998; Ross *et al.*, 2002), TS females suffer from deficits in a range of

cognitive]domains, notably visuospatial skills and working memory (Buchanan *et al.*, 1998; Bishop *et al.*, 2000), facial affective recognition (Lawrence *et al.*, 2003; Skuse *et al.*, 2005) and memory (Murphy *et al.*, 1994; Murphy *et al.*, 1997), and executive function (Ross *et al.*, 1995; Temple *et al.*, 1996; Romans *et al.*, 1997; Kirk *et al.*, 2005). In terms of executive function, TS females took longer to respond in the Stroop task (Temple *et al.*, 1996) and in a similar contingency naming test (which eliminates the confound of varying reading levels; Kirk *et al.*, 2005). The human Stroop test requires subjects to read the colour of the ink in which a colour word is written; for example, the stimuli 'BLUE' and 'GREEN' will have 'blue' and 'red' as correct answers. Whilst the congruent stimulus 'BLUE' does not involve response conflict (i.e. both ink colour and word meaning are 'blue'), the incongruent stimulus 'GREEN' requires the subject to inhibit the prepotent and incorrect response of 'green', because humans, due to practice effects, tend to say the word meaning rather than the ink colour. One can index behavioural inhibition by measuring response time to congruent and incongruent stimuli. Furthermore, TS girls showed impaired performance on the Rey-Osterrieth organisational and Tower of Hanoi tests, with higher levels of impulsivity, compared to control subjects (Romans *et al.*, 1997). TS females have also shown abnormal prefrontal cortex function during response inhibition in a functional magnetic resonance imaging study (Tamm *et al.*, 2003).

The purpose of this chapter is to utilise the XO mouse model to examine the possibility of any X-monosomy and/or X-linked parent-of-origin effects on performance on a biconditional discrimination and response conflict task modified for rodent use, which mimicked some aspects of response competition seen in the Stroop test. The rationale for the use of this task is: (i) there is an element of behavioural inhibition, and the role of orbitofrontal cortex has been implicated (Szatkowska *et al.*, 2007), in both this task and the previous reversal learning study (Davies *et al.*, 2005a) in which an X-linked parent-of-origin effect was found, (ii) in many ways this task is analogous to the human Stroop test in which TS females have shown impaired performance and (iii) this task involves executive function, and TS females have shown aberrant prefrontal cortex functions which might underlie certain deficits in executive functions. In this task, mice were required to learn two instrumental biconditional discriminations, one auditory and one visual, in two different contexts. At test, subjects were presented with audiovisual stimulus compounds in each of the two training contexts; these compounds were comprised of either training stimulus elements that both dictated the same instrumental response during training (i.e. congruent), or of training stimulus elements that dictated different instrumental responses (i.e. incongruent). In the incongruent situation, the subject would need to use the contextual cues to disambiguate the conflicting stimulus elements. Congruent and incongruent types were analogous to situations in

the Stroop test where the subject received 'BLUE' and 'GREEN' stimuli, respectively, with the latter involving a response competition. The biconditional discrimination task is non-spatial, which suits my present purposes as I would like to examine specifically biconditional discrimination learning and response conflict without the need for spatial ability, as TS females (and therefore the XO mice might) display visuospatial impairments given the possibility of X-monosomy effects on visuospatial function (Buchanan *et al.*, 1998; Bishop *et al.*, 2000) which could confound the interpretation of results.

Biconditional discrimination and response conflict tasks in rats have been published upon previously (Haddon & Killcross, 2005, 2006a, 2006b; Haddon *et al.*, 2008; Marquis *et al.*, 2007); however, this task has not been published using mice. It has been shown with normal rats that they could acquire the two biconditional discrimination pairs successfully, and at test, to use contextual cues to dictate their responses correctly. Animals could successfully complete both congruent and incongruent trial types, achieving significantly more correct than incorrect responses; however, the difference between correct and incorrect responses was smaller in the incongruent trials due to interference between the two stimulus-response associations that were competing for two different responses (Haddon *et al.*, 2008). Various task manipulations have been shown to affect performance; for example, using differential training between contexts, animals could successfully complete the incongruent trial types in the overtrained context but not in the undertrained context, suggesting that interference from the overtrained context was negatively affecting performance in the undertrained context (Haddon & Killcross, 2006b). Numerous neurobiological factors underlying performance on the biconditional discrimination and response conflict have been identified using lesion studies in specific brain regions; large lesions to the prefrontal cortex, encompassing prelimbic and anterior cingulate cortices, led to animals failing to respond correctly in incongruent trials (Haddon & Killcross, 2005, 2006a). Further examination and work into prefrontal cortex lesions have found that when the prelimbic cortex was specifically damaged, animals were impaired on incongruent trials, suggesting the prelimbic cortex allows for the use of task-setting cues to guide goal directed behaviour (Marquis *et al.*, 2007). In cases when the anterior cingulate cortex was lesioned, animals were impaired during the first ten seconds of the incongruent trials, which suggests the anterior cingulate cortex is involved in the detection of response conflict (Haddon & Killcross, 2006a). Lesions of the hippocampal formation in rats, surprisingly, resulted in better performance in incongruent trials; however, whilst these animals showed an influence of specific reinforcer devaluation on instrumental performance (indexed by lever presses), they did not on Pavlovian performance (indexed by magazine approaches; Haddon & Killcross, 2007).

As introduced in Chapters I and III, the XO mouse model allows dissociations between effects due to the parental origin of the single X chromosome and X-monosomy effects. Differences between 39,X^mO and 39,X^PO animals could point to an X-linked parent-of-origin effect, whereas differences between 39,XO and 40,XX animals could point to an X-monosomy effect. Given that 39,X^mO mice and 45,X^m TS females had been shown to perform worse in tasks taxing response conflict (Davies *et al.*, 2005a) and behavioural inhibition (Skuse *et al.*, 1997) respectively, one might predict that the 39,X^mO mice would perform worse than 39,X^PO and 40,XX animals in the response conflict task.

However, the 39,X^mO and 39,X^PO animals were generated using two different crosses with different mothers and littermates, and thus it was considered necessary to control for the possibility by testing 40,XX^{Paf} and 40,$X^{Paf}X$ animals (i.e. the resulting female littermates with the normal XX karyotype of the two generating crosses) alongside the 39,X^mO, 39,X^PO and 40,XX animals. As explained in Chapter III, this possible confound stemming from two different generation crosses does not apply to where X-monosomy effects are detected, since in that case, effects are common to both 39,X^mO and 39,X^PO animals. Therefore, the data from the 40,XX^{Paf}, 40,$X^{Paf}X$ comparisons (the *Paf* comparison) were only presented in the Results section when there was an apparent parent-of-origin effect in the XO comparison.

4.2 Materials and methods

4.2.1 Subjects and animal husbandry

In this experimental chapter, animals from the XO mouse model (39,X^{m}O, 39,X^{P}O and 40,XX) and associated female littermates (40,XX^{Paf} and 40,X^{Paf}X) were subject to biconditional discrimination testing. Subjects were aged six months at the start of testing. **Table 4.2.1i** shows the number of subjects tested in this task. XO generating crosses were detailed in the General Introduction and in Chapter III, **3.2.1.** General housing, handling and behavioural testing conditions were as described in Chapter II, **2.2** and **2.3.**

Table 4.2.1i Numbers of subjects (n) tested in the biconditional discrimination task across the training phases. Numbers in parentheses referred to the total number of animals tested, including those subsequently excluded as they progressed through the training from reinforcer preference to probe sessions at the end of the experiment (see **4.2.5** for exclusion criteria).

Test	Karyotype and 'n'				
	39,X^{m}O	39,X^{P}O	40,XX	40,XX^{Paf}	40,X^{Paf}X
Reinforcer preference	15	12	9	14 (15[1])	10
Magazine training	15	11 (12)	9	15	10
Nose poke training	15	12	9	15	10
Biconditional discrimination training	13 (15)	10 (12)	7 (9)	11 (15)	7 (10)
Probe sessions	13 (15)	10 (12)	5 (9)	11 (15)	6 (10)

[1]: one subject was excluded from the final maltodextrin vs. sucrose preference analysis due to being an outlier; the subject was included in previous maltodextrin vs. water and sucrose vs. water analyses.

4.2.2 Body weight and oestrus status

The body weights of subjects were monitored regularly for signs of ill health and dehydration. This was particularly important as subjects were placed on the home water restriction schedule (see Chapter II, **2.5**) for the duration of the experiment. The oestrus status of female subjects was determined by vaginal smearing. Smearing was performed after testing, on the first seven days of both biconditional discrimination training and probe testing. Details of the procedure can be found in Chapter II, **2.4.**

4.2.3 Reinforcer preference test

In advance of testing on the main biconditional discrimination task all the subjects were tested for their reactivity and preference to the liquid reinforcers used to motivate performance in the task. Two weeks prior to the reinforcer preference test subjects were placed on the home water restriction schedule (Chapter II, **2.5**) and then habituation to, and preference for, the reinforcers, 10% maltodextrin solution (flavoured with grape; w/v 0.05% Kool-Aid flavouring, Kraft Foods Inc.) and 10% plain sucrose solution, was assessed. General information about this procedure can be found in Chapter II, **2.6**. For preference testing of 2 distinct reinforcers, the procedure was slightly modified from that described in Chapter II. Animals were tested for a period of 10 days. On the first 2 days, animals were habituated to the testing environment and exposed to only water. For the next 6 days, animals were given 2 containers, one filled with water and the other with either maltodextrin or sucrose in a pseudorandomised order, so that animals were exposed to each of the reinforcer for 3 days. For the last 2 days, animals were exposed to both maltodextrin and sucrose solutions (no water was present). Percentages preference for maltodextrin vs. water, sucrose vs. water and maltodextrin vs. sucrose were calculated.

4.2.4 Biconditional discrimination task

Upon completion of the reinforcer preference testing, animals began the biconditional discrimination task using the apparatus described in Chapter II, **2.7.4**. The task consisted of the following phases: (1) magazine training, (2) nose poke training, (3) biconditional discrimination training, and (4) probe sessions.

4.2.4.1 General experimental design of the task

Mice were trained on two biconditional discrimination tasks (one auditory, one visual) in two different contexts (C1 and C2), with two different reinforcers (R1 and R2). In each of the two biconditional discrimination tasks, there were two auditory (A1 and A2) or two visual (V1 and V2) cues (**Table 4.2.4.1i**). In C1, when auditory cue A1 was presented, mice were reinforced when they made the response of nose poke NP1 (but not reinforced when they made the nose poke NP2) and when auditory cue A2 was presented, nose poke NP2 would result in reinforcement (but not nose poke NP1). Animals in C1 were rewarded with reinforcer R1. In C2, when visual cue V1 was presented, nose poke NP1 would result in reinforcement (but not nose poke NP2) and when visual cue V2 was presented, animals were reinforced after nose poke NP2 (but not after nose poke NP1). Animals in C2 were rewarded with reinforcer R2. The usage of a particular reinforcer in each context helped to strengthen the contextual learning in

subjects. Animals were trained on both contexts each day; one context in the morning and the other in the afternoon. In order to control for possible differences in motivation between the morning and afternoon sessions, there was at least a four-hour gap between the two sessions.

Table 4.2.4.1i Experimental design of the biconditional discrimination task, and specific stimulus elements, contexts and reinforcers used in the current experiment. Nose poke responses outlined in the training phase columns are the correct and reinforced response. Nose poke responses outlined in 'Probe trials' columns are the correct responses given the particular congruent and incongruent stimulus compounds. The yellow highlighted stimulus elements within the incongruent stimulus compounds are those that dictate the context-appropriate responses.

	Training (to criterion)			Probe trials (done in extinction)	
Context	Nose poke NP1	Nose poke NP2	Reinforcer	Congruent	Incongruent
C1	A1	A2	R1	A1V1 →NP1	A1V2 →NP1
				A2V2 →NP2	A2V1 →NP2
C2	V1	V2	R2	A1V1 → NP1	A1V2 →NP2
				A2V2 → NP2	A2V1 →NP1

	Specific stimulus elements, contexts and reinforcers used in this experiment
Contexts	Grid floor and smooth floor
Auditory	Tone and buzz
Visual	House and stimulus lights
Nose poke responses	Left and right nose pokes
Reinforcer	10% grape-flavoured maltodextrin and 10% plain sucrose solutions

After animals had reached criterion performance (see details below) on the two biconditional discrimination tasks, subjects were put through probe trials (done in extinction) in which they were presented with audiovisual compound cues, in each of the contexts C1 and C2. These audiovisual compounds could be made up of either training stimulus elements that were linked to the same reinforced nose poke response in the training phase (i.e. A1V1, both linked to NP1 response for reinforcement and A2V2, both linked to NP2 response for reinforcement), or

training stimulus elements that were linked to different reinforced nose poke responses (i.e. A1V2 and A2V1). These were termed congruent and incongruent stimulus compounds respectively. As the incongruent stimulus compounds were linked to conflicting nose poke responses, animals would need to use context to guide behaviour. For example, if the subject was presented with an incongruent compound A1V2 in context C1, then the context-appropriate response would be NP1, because during the training period in context C1, subjects would have been reinforced on the NP1 response following an A1 stimulus cue. All subjects were counterbalanced across all the elements within the task.

4.2.4.2 Magazine training

Subjects were trained to retrieve the reinforcer from the magazine. Animals received two training sessions on one day, one in the morning (with context C1 and reinforcer R1) and one in the afternoon (with context C2 and R2). A single training session was 40 minutes long. Reinforcer was available on a RI-60 (random interval) schedule (i.e. animals were given an reinforcer on average every 60 seconds). There was an IRI (inter-reinforcement interval) of 60 seconds (i.e. there is at least 60 seconds in between each availability of reinforcement). During each training session, there were no visual (house/stimulus) or auditory (tone/buzz) stimuli, and no nose poke lights. The number of magazine entries, the total duration of the magazine entries and the number of reinforcers drunk were noted.

4.2.4.3 Nose poke training

Subjects were trained to nose poke the nose poke aperture when its light was on. Either the left or the right nose poke aperture light was on at any one time, and the animal was required to poke into the illuminated aperture (a correct response) in order to receive reinforcement. Sessions were twice daily, one in the morning (with context C1 and reinforcer R1) and one in the afternoon (with context C2 and reinforcer R2). Each session lasted for 26 minutes and consisted of eight trials (four left and four right nose poke illuminations, lit in a pseudorandom order[10]). Each trial consisted of 10 seconds of pre-CS period (CS: conditioned stimulus, i.e. nose poke lights), followed by a 120 second of nose poke light illumination and reinforcement period.

[10] The pseudorandom order was such that neither left nor right nose poke light would be illuminated more than three times in a row.

There was a variable ITI (inter-trial interval[11]) before beginning with 10 seconds of pre-CS period on the next trial.

During the reinforcement period, subjects were initially reinforced on a CRF (continuous reinforcement) schedule, in which every time a correct response was made, the animal was reinforced. As training progressed, the schedule was moved to RI-5, RI-10 and finally RI-15 (RI-X schedule: reinforcement was provided for the first correct response after an average of X seconds since the last reinforcement) where the reinforcement schedule remained for the rest of the biconditional discrimination task. Before moving onto a next leaner schedule, animals were required to fulfil a nose poke training performance criterion of making enough correct nose poke responses to receive a total of 30 or more reinforcements daily (total reinforcement for the two sessions), to ensure that they had learnt to nose poke correctly at a competent rate.

4.2.4.4 Biconditional discrimination training

Upon completion of nose poke training, biconditional discrimination training began. During the training, both nose poke lights were illuminated. Subjects were required to make the correct nose poke response based on the auditory or visual stimulus cue in a particular context, in order to receive reinforcement (**Table 4.2.4.1i**). Two sessions per day were given, one in the morning (with context C1 and reinforcer R1, exposed to either auditory or visual stimulus cues) and one in the afternoon (with context C2 and reinforcer R2, exposed to the stimulus cues previously not trained in the morning). Each session lasted for 26 minutes and consisted of eight trials (four tone and four buzz stimulus cues, or four house light and four stimulus lights stimulus cues, presented in a pseudorandom order[12]). Each trial consisted of a 10 second pre-CS period, followed by a 120 second of presentation of stimulus cues and reinforcement on a RI-15 schedule after the animal had made correct nose poke responses. There was a variable ITI (inter-trial interval[13]) before beginning with 10 seconds of pre-CS period on the next trial (**Figure 4.2.4.4a**).

Subjects' performance was indexed by CS' (CS prime) values and their derivatives. CS' correct values indicated the number of correct nose poke responses made before any reinforcement was given at the start of a trial within a session; likewise, CS' incorrect values were the number of

[11] The ITI was randomly chosen from this list of durations: 40, 45, 50, 50, 55, 55, 60, 60 seconds.

[12] The pseudorandom order was such that none of the stimulus cues would be presented more than twice in a row.

[13] The ITI was randomly chosen from this list of durations: 40, 45, 50, 50, 55, 55, 60, 60 seconds.

incorrect nose poke responses made before reinforcement within a trial. The CS' measure was an unbiased index of discrimination performance (as opposed to CS correct / incorrect measure, which was the total correct / incorrect nose poke responses throughout the trial, not restricted to the first non-reinforced period as indexed by CS'), as after a reinforcement had been given, the subject could 'track' the reinforcer, that is, repeating the nose poke response it made before reinforcement, without truly learning the discrimination rules[14]. CS' measure would remove the confounding 'tracking' behaviour which might otherwise be present in the CS measure. CS' correct and CS' incorrect values for the four stimulus cues (house light, stimulus lights, tone and buzz) over the relevant trials in a given session were averaged to give mean values of CS' correct and mean CS' incorrect, and discrimination ratio was calculated following the formula:

$$\text{Discrimina tion ratio} = \frac{\text{mean CS'correct}}{\text{mean CS'correct} + \text{mean CS'incorrect}}$$

The four discrimination ratios for each of the stimulus cues were averaged over five sessions, and then averaged over the four stimulus cues to give a single value. In order for subjects to successfully complete biconditional discrimination training, the final discrimination value would need to be 0.63 or higher. For further confirmation that the subjects had learnt the relevant nose poke response for each of the four stimulus cues, the average CS' correct and CS' incorrect values of each stimulus cues for the five criterion sessions were examined to ensure that CS' correct was consistently higher than CS' incorrect for each stimulus cues over the five sessions.

4.2.4.5 Compound stimuli probe sessions

After animals had acquired the biconditional discrimination, they were given eight days of probe sessions. There were two sessions daily (a total of 16 probe sessions were given), one in the morning and one in the afternoon. A single probe session consisted of 12 trials, which were a combination of four probe trials which were done in extinction and eight normal reinforced trials (identical to those given in biconditional discrimination training); this was to avoid animals

[14] For example, at the start of each trial, the animal could be responding left, right, left, right, and so on. Reinforcement was given after an average of 15 seconds (RI-15 schedule) and a correct response (e.g. left) from the animal. The animal then realized the correct response was left because it was just reinforced on it, and continued to respond left for the rest of the trial without learning that e.g. a house light stimulus cue required a left nose poke response.

becoming de-motivated by the lack of reinforcement and disconcerted due to the sudden appearance of probe trials. The trials were presented in a pseudorandom order.[15]

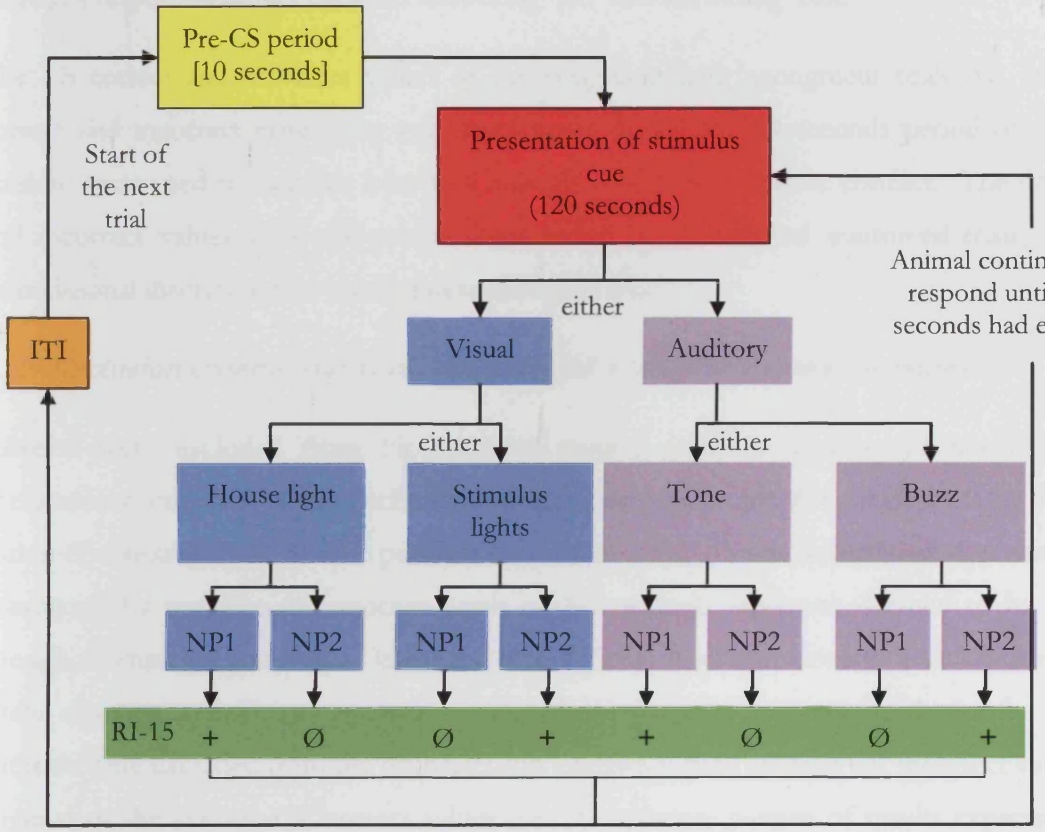


Figure 4.2.4.4a Schematic diagram showing the event flow within a trial in a biconditional discrimination training session. After a 10 second pre-CS period, there was a 120-second presentation of the stimulus cue during which a subject responded continuously to obtain reinforcement at a RI-15 schedule. Following this, there was a variable ITI after which another trial began. + symbol signifies reinforcement after a correct response while Ø indicates no reinforcement after an incorrect response.

The four probe trials within the session consisted of two congruent and two incongruent trials (see above, section **2.4.1**), which were either (1) tone & stimulus lights, (2) tone & house light, (3) buzz & stimulus lights and (4) buzz & house light. Whether these stimulus cue compounds were congruent or incongruent depended on the training contexts and the current contexts in which the animals were probed. Probe trials were 30 seconds in length and no reinforcement was given

[15] Such that there would not be more than two probe trials in a row, and normal trials would not have any of the stimulus cues presented more than twice in a row.

during the period. These probe trials are analogous to the 'Stroop' test in humans; the incongruent trials in this present study are similar to the situation with a colour word that is written in a different ink colour to its meaning (e.g. RED, correct answer would be 'blue'), as there is a response conflict between answering 'red' and answering 'blue'.

The CS correct and incorrect values of the congruent and incongruent trials (i.e. number of correct and incorrect nose poke responses made during the 30 seconds period of the probe session) were used to examine how well animals cope with response conflict. The CS' correct and incorrect values were the performance index for the normal reinforced trials, as in the biconditional discrimination training described previously.

4.2.5 Exclusion criteria, statistical analysis and additional control measures

Subjects were excluded from biconditional training analyses if they did not achieve the performance criterion (final discrimination ratio, averaged over five days, of 0.63 or higher) within 60 training sessions. The performance criterion of 0.63 was determined after a pilot study conducted by myself using another batch of XO animals; 0.63 was deemed to be stringent enough to ensure animals have learnt the biconditional discriminations to a satisfactory degree, while allowing a sufficient number of animals to progress through to the probe sessions. Animals were excluded from the probe session analyses if their average CS' incorrect values were larger than the average CS' correct values (i.e. the opposite pattern of results expected), taken from normal reinforced trials within the probe sessions. The normal reinforced trials in the probe sessions indicated the general capability to perform the discrimination task, and if the subject was failing the simple discrimination, it was unlikely that the results on the congruent and incongruent trials would reflect properly the subject's ability to deal with response conflict. Data points which were deemed extreme outliers (on a box plot, data points that lay 3 times the interquartile range from the hinges were extreme outliers), as calculated by SPSS, were excluded.

Statistical analyses were performed using SPSS software (version 17, SPSS Inc., IBM, U.S.A.). Data were subject to One Way ANOVA. As noted in Chapter III, **3.2.5**, two comparisons were made, with Comparison 2 only performed where an X-linked parent-of-origin effect was apparent in Comparison 1.

Comparison 1: 'XO comparison' (Between Subjects factor KARYOTYPE): 39,$X^{m}O$, 39,$X^{p}O$, 40,XX. The purpose of this main comparison was to check for effects of X-monosomy and X-linked parent-of-origin effects.

Comparison 2: '*Paf* comparison' (Between Subjects factor GENOTYPE): 40,XX, 40,XX^{Paf}, 40,X^{Paf}X. An additional comparison was made between females from the 39,X^{m}O-generating cross (40,XX^{Paf}) and females from the 39,X^{p}O-generating cross (40,XX and 40,X^{Paf}X). The purpose of this second comparison was to check for gross cross effects only where differences between 39,X^{m}O and 39,X^{p}O mice were found.

Repeated Measures ANOVA was used when appropriate; additional factors specific to the analysis were defined in the relevant Results section, and furthermore, Greenhouse-Geisser (epsilon of 0.75 or lower) or Huynh-Feldt (epsilon of 0.75 or higher) corrections were applied to degrees of freedom if the Mauchly's Test of Sphericity was violated in Repeated Measure tests. When initial ANOVA revealed a significant effect, Tukey HSD Test or Dunnett T3 Test (in the cases with unequal variances) was performed for *post hoc* comparisons, and when a significant interaction was revealed, Least Significant Difference adjustment was used for *post hoc* pairwise comparisons. Kruskal-Wallis One Way ANOVA and Friedman test (non parametric repeated measures test) were used for data not normally distributed. One sample t-test was used when comparing a set of data against a hypothetical value. For all comparisons, p values of <0.05 were regarded as significant.

4.3 Results

4.3.1 Reinforcer preference test

The preference for 10% grape flavoured maltodextrin solution reinforcer (**Figure 4.3.1a**) over water and preference for 10% plain sucrose solution reinforcer over water (**Figure 4.3.1b**) both increased over the testing period. Data on percentage preference for reinforcer over water were subject to Repeated Measures ANOVA with Between Subject factor of KARYOTYPE (i.e. 39,X^mO, 39,X^pO and 40,XX) and Repeated Measures factor of DAY (i.e. first, second and third day of exposure to maltodextrin). The 39,X^mO, 39,X^pO and 40,XX animals did not differ significantly from each other in terms of their percentage preference for maltodextrin over water over the three days of maltodextrin exposure (effect of KARYOTYPE, $F_{2,33} = 2.62$, n.s.). As expected, the preference for maltodextrin increased significantly over the three days of exposure (effect of DAY, $F_{2,66} = 49.085$, $p<0.001$) and there were no interactions between KARYOTYPE and DAY ($F_{4,66} = 0.993$, n.s.).

Figure 4.3.1a

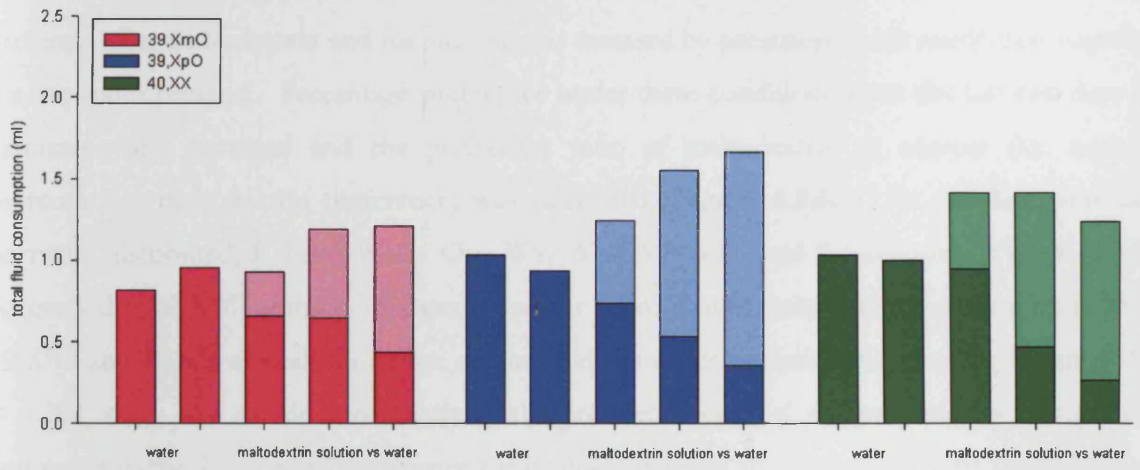


Figure 4.3.1a Total fluid consumption, as a proportion of 10% grape flavoured maltodextrin solution reinforcer over water intake in the XO comparison. Preference for maltodextrin (light bars) over water (dark bars) increased over this period. Note that on days 1 and 2, only water was available. Data were presented as mean values.

Similarly, 39,X^mO, 39,X^pO and 40,XX animals did not differ in their preference for sucrose solution over water during the three days of exposure to the reinforcer (effect of KARYOTYPE, $F_{2,33} = 2.675$, n.s.; **Figure 4.3.1b**). The preference increased significantly over the testing period (effect of DAY, $F_{2,66} = 12.625$, $p<0.001$) and there was no interaction between KARYOTYPE and DAY ($F_{4,66} = 0.432$, n.s.).

Figure 4.3.1b

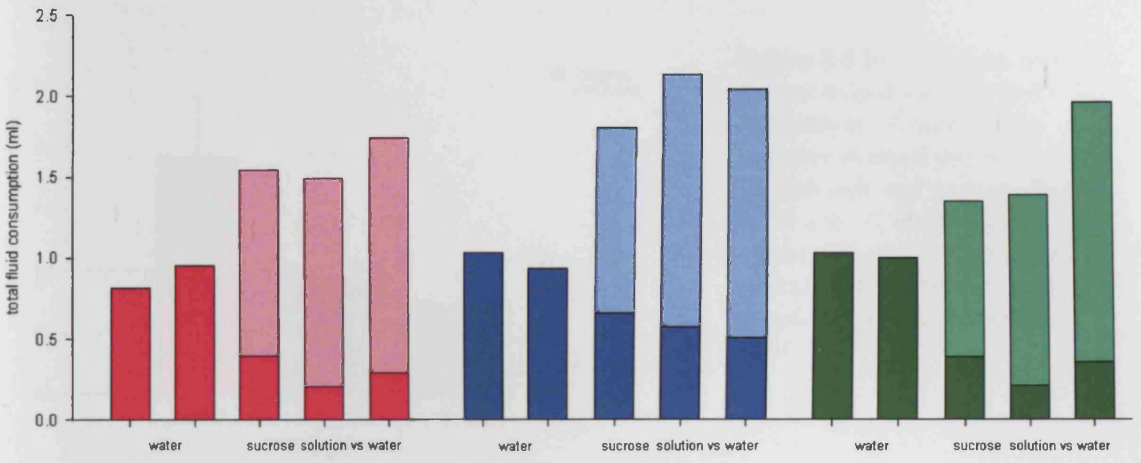


Figure 4.3.1b Total fluid consumption, as a proportion of 10% plain sucrose solution reinforcer over water intake in the XO comparison. Preference for sucrose (light bars) over water (dark bars) increased over this period. Note that on days 1 and 2, only water was available. Data were presented as mean values.

In an additional manipulation at the end of the reinforcer vs. water choice assay, the relative preference for maltodextrin and for sucrose was assessed by presenting both reinforcers together (i.e. no water present). Percentage preference under these conditions from the last two days of exposure were averaged and the preference ratio of maltodextrin to sucrose (i.e. sucrose preference ÷ maltodextrin preference) was calculated (**Figure 4.3.1c**). As the data were not normally distributed, Kruskal-Wallis One Way ANOVA was used for analysis. **Figure 4.3.1c** suggests that 39,$X^{m}O$ animals appeared to prefer sucrose over maltodextrin solution more than 39,$X^{p}O$ and 40,XX animals; however, animals did not differ in their preference significantly (H_2 = 5.294, n.s.). As an additional analysis, the preference ratio of maltodextrin to sucrose was compared to the 1 (i.e. equal preference for maltodextrin and sucrose) to examine the possibility of reinforcer preference biases in a particular group of animals, by conducting a simple one-sample t-test for each of the karyotype groups. No significant differences between the reinforcer preference ratio and the ratio of 1 were observed in any of the karyotype groups (39,$X^{m}O$: t_{14} = 1.87, n.s.; 39,$X^{p}O$: t_{11} = -0.104, n.s.; 40,XX: t_8 = -1.333, n.s.).

Figure 4.3.1c

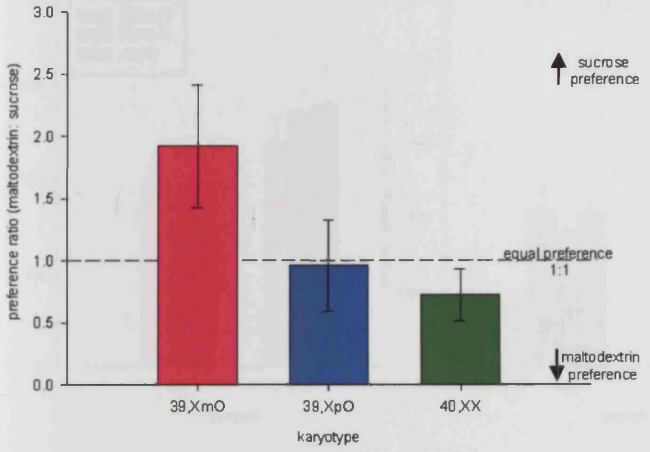


Figure 4.3.1c Preference ratio of sucrose to maltodextrin in the XO comparison. A ratio of one indicates an equal preference for maltodextrin and sucrose. Ratios of >1 and <1 indicate preference for sucrose and preference for maltodextrin respectively. Data are presented as mean values ± SEM.

4.3.2 Magazine training

(i) Total number and total duration of magazine entries:

Data on the single day of magazine training (**Figure 4.3.2a**) were subject to analysis by Repeated Measures ANOVA, with Between Subject factor of KARYOTYPE and Repeated Measure factor of TIME (i.e. AM and PM sessions). For 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX animals, the total number of magazine entries and the total duration of the entries increased significantly in the PM session compared to the AM session (magazine entries, effect of TIME, $F_{1,32} = 17.867$, $p<0.001$; duration, effect of TIME, $F_{1,32} = 28.621$, $p<0.001$), suggesting the animals had successfully acquired the association between the magazine and reinforcer delivery. In the second magazine training session in the PM, animals were making on average 100 – 150 magazine entries, which was an excellent rate of response. Furthermore, there was no group differences in the acquisition of this association, as indicated by non-significant effect of KARYOTYPE in both magazine entries ($F_{2,32} = 0.248$, n.s.) and duration ($F_{2,32} = 0.140$, n.s.), and no interaction between KARYOTYPE and TIME in magazine entries ($F_{2,32} = 1.254$, n.s.) and duration ($F_{2,32} = 1.69$, n.s.).

Figure 4.3.2a

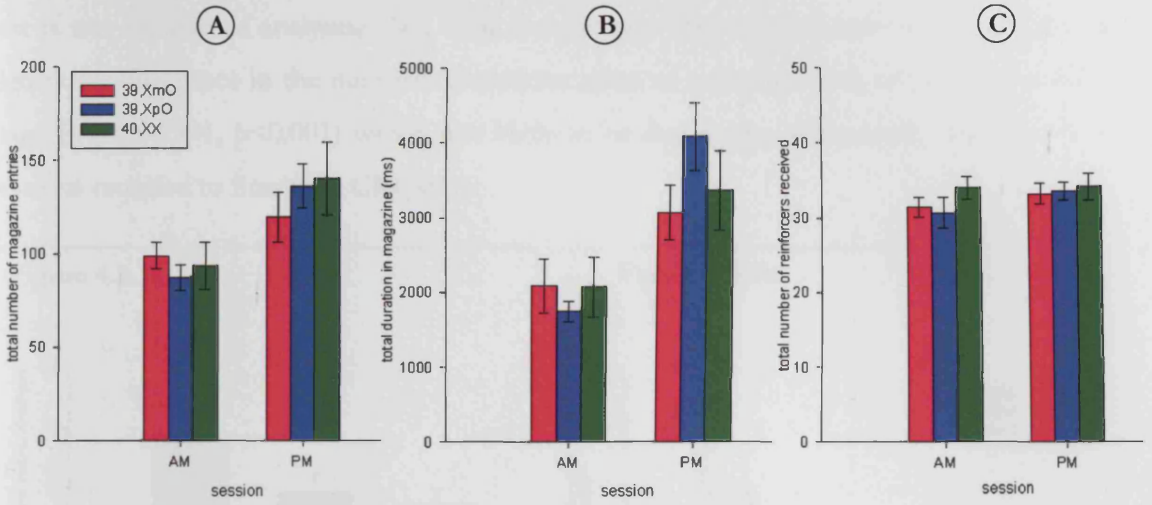


Figure 4.3.2a [A] Total number of magazine entries, [B] total duration in magazine and [C] total number of reinforcers received during the two magazine training sessions in the XO comparison. Data are presented as mean values ± SEM.

(ii) Total number of reinforcers received:

For 39,X^{m}O, 39,X^{p}O and 40,XX animals comparison, there were no group differences in the number of reinforcers received (**Figure 4.3.2a**; effect of KARYOTYPE, $F_{2,32} = 2.069$, n.s.) and no interaction between TIME and KARYOTYPE ($F_{2,32} = 0.413$, n.s.). The reinforcers were delivered on a RI-60 schedule on both AM and PM sessions and so there was a cap on the quantity of reinforcers received, and there were no differences in number of reinforcers received between AM and PM sessions (effect of TIME, $F_{1,32} = 1.586$, n.s.), i.e. in the presence of similar number of reinforcers, animals were responding more in the PM than AM which indicates that they had been successfully trained.

4.3.3 Nose poke training

The total number of sessions taken to complete nose poke training (**Figure 4.3.3a**) did not differ significantly between the 39,X^{m}O, 39,X^{p}O and 40,XX animals (effect of KARYOTYPE, $F_{2,33} = 2.518$, n.s.). **Figure 4.3.3.b** shows the numbers of sessions taken in reaching CRF, RI-5, RI-10 and RI-15 reinforcement schedule stages, as the animals progressed through training. The karyotype groups took similar number of sessions to complete each stage, with the CRF stage taking most number of sessions, reflecting the initial formation of association between nose poke aperture illumination and nose poke response, in order to receive reinforcement. As these data were not normal, a Repeated Measures ANOVA was not computed and instead a non-parametric Friedman test was used to analyse the data, with Repeated Measures factor of

SCHEDULE (i.e. CRF, RI-5, RI-10 and RI-15 reinforcement schedule) only, as the Friedman test is not capable of analysing data with two factors. The analysis indicated that there was a significant difference in the number of sessions taken to complete each reinforcement schedule stage ($\chi^2_3 = 54.991$, $p<0.001$) which was likely to be due to the substantially higher number of sessions required to finish the CRF stage.

Figure 4.3.3a

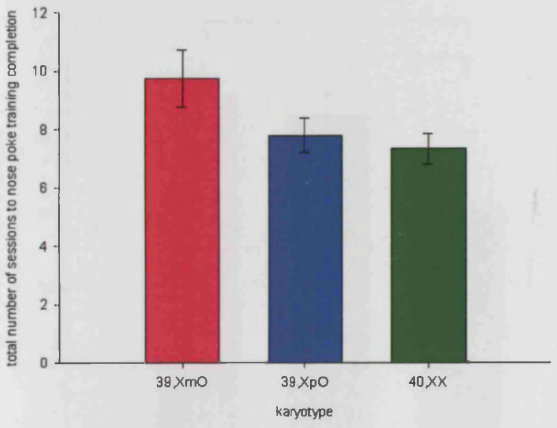


Figure 4.3.3b

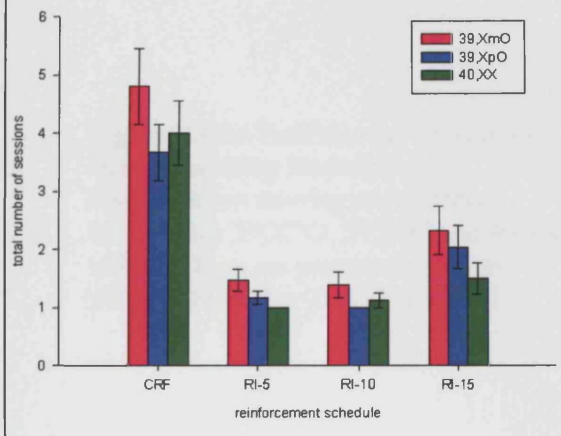


Figure 4.3.3a Total number of sessions taken in order to complete nose poke training successfully in the XO comparison. Data are presented as mean values ± SEM.

Figure 4.3.3b Total number of sessions taken to achieve the performance criterion (enough correct nose poke responses to receive 30 reinforcements) of completing nose poke training at the four reinforcement schedule stages (CRF, RI-5, RI-10, RI-15) in the XO comparison. Data are presented as mean values ± SEM.

4.3.4 Biconditional discrimination training

(i) Total number of sessions to reach performance criterion:

To complete biconditional discrimination training, animals were required to fulfil the performance criterion of achieving a final discrimination ratio of 0.63 or higher (see **Section 4.2.4.4**). The total number of sessions taken in reaching biconditional discrimination training performance criterion (**Figure 4.3.4a**) differed significantly between 39,X^mO, 39,X^pO and 40,XX subjects (effect of KARYOTYPE, $F_{2,27} = 7.27$, $p<0.01$). There were 2 out of 15 39,X^mO, 2 out of 12 39,X^pO and 2 out of nine 40,XX mice which did not achieve this criterion within 60 sessions, suggesting that the task, as conducted here, was relatively difficult for mice (**Table 4.2.1i**); data from these mice were not included in the above analysis (see exclusion criteria **4.2.5**). As variances were not equal, the data were subject to the Dunnett T3 *post hoc* test, which found

the performance of 40,XX animals to differ significantly from that of 39,X^{m}O ($p<0.001$) and 39,X^{p}O ($p<0.02$) animals; 40,XX animals took significantly fewer number of sessions to complete biconditional discrimination training than 39,X^{m}O and 39,X^{p}O animals, whose performance did not differ significantly from each other. This result suggests there was an X-monosomy effect on the speed of task acquisition; the lack of an X chromosome, regardless of its parental origin, was detrimental to task acquisition.

Figure 4.3.4a

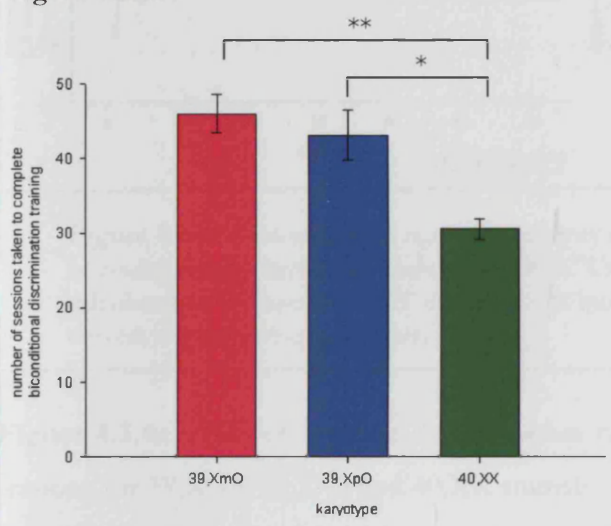


Figure 4.3.4a Total number of sessions taken in reaching biconditional discrimination training performance criterion for 39,X^{m}O, 39,X^{p}O and 40,XX animals. Data are presented as mean values ± SEM. * $p<0.02$, ** $p<0.001$.

(ii) Relative performance in auditory and visual modalities during biconditional discrimination training:

In order to see whether the overall slower learning of the biconditional task was due to interactions between karyotype and the particular sensory modality used in the learning, the data were separated by auditory or visual stimulus modality (i.e. by buzz and tone together and by house light and stimulus lights together, respectively) across sessions. The performance was indexed by the discrimination ratio, which was calculated by $\frac{CS'correct}{CS'correct + CS'incorrect}$. A ratio higher than 0.5 would signify more correct than incorrect responses. Repeated Measures ANOVA, with Within Subject factor SESSION (sessions 1 to 60 in the 39,X^{m}O, 39,X^{p}O and 40,XX comparison; animals which had completed biconditional discrimination training before session 60 were given the score of their last discrimination ratio until session 60) and Between Subject factor KARYOTYPE (comparison of 39,X^{m}O, 39,X^{p}O, 40,XX) was used to analyse the data.

Figure 4.3.4c

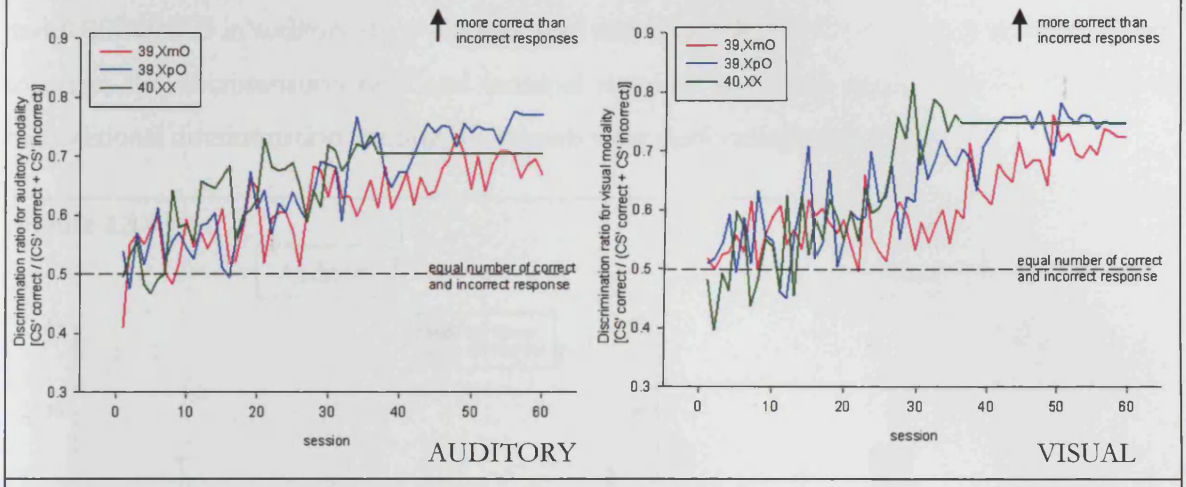


Figure 4.3.4c Discrimination ratios for auditory and visual modalities, across sessions, during biconditional discrimination training for 39,X^mO, 39,X^pO and 40,XX animals. Ratios were calculated by CS' correct ÷ (CS' correct + CS' incorrect). Ratios over 0.5 would signify more correct than incorrect responses.

Figure 4.3.4c shows the average discrimination ratios for auditory and visual modalities across sessions for 39,X^mO, 39,X^pO and 40,XX animals. For the auditory modality, subject groups did not differ significantly in their performance across the training phase (effect of KARYOTYPE, $F_{2,27} = 0.986$, n.s.). Performance improved significantly across SESSION ($F_{59,1593} = 5.971$, $p<0.001$) which reflects the subjects' acquisition of the auditory stimulus-response associations over time; however, this improvement across session did not vary between karyotype groups (SESSION × KARYOTYPE, $F_{118,1593} = 0.801$, n.s.). Similarly for the visual modality, subject groups did not differ significantly in their performance across sessions (effect of KARYOTYPE, $F_{2,27} = 1.578$, n.s.). Once again, performance improved significantly over SESSION ($F_{59,1593} = 7.783$, $p<0.001$), reflecting acquisition of visual stimulus-response associations, and this improvement did not vary between groups (SESSION × KARYOTYPE, $F_{118,1593} = 1.179$, n.s.).

In addition to the ratio data above, terminal response rates for both auditory and visual modalities (i.e. CS' correct and incorrect for each karyotype group at the end of biconditional discrimination training) are shown in **Figure 4.3.4d**. Data were analysed using Repeated Measures ANOVA, with Within Subject factor RESPONSE (i.e. correct or incorrect CS') and Between Subject factor KARYOTYPE (i.e. comparison of 39,X^mO, 39,X^pO, 40,XX). For both auditory and visual modalities, there were significantly more correct than incorrect RESPONSES (auditory: $F_{1,27} = 36.155$, $p<0.01$; visual: $F_{1,27} = 11.16$, $p<0.01$). There were no differences between 39,X^mO, 39,X^pO and 40,XX karyotype groups in terms of performance; there were no

significant main effect of KARYOTYPE in either auditory ($F_{2,27}$ = 2.404, n.s.) or visual ($F_{2,27}$ = 2.15, n.s.) modalities. Furthermore, there was no significant interaction between KARYOTYPE and RESPONSE in auditory ($F_{2,27}$ = 0.820, n.s.) and visual ($F_{2,27}$ = 1.917, n.s.) modalities. Taken together, the discrimination ratio and terminal response rates data suggest that by the end of biconditional discrimination training, all animals were performing equivalently.

Figure 4.3.4d

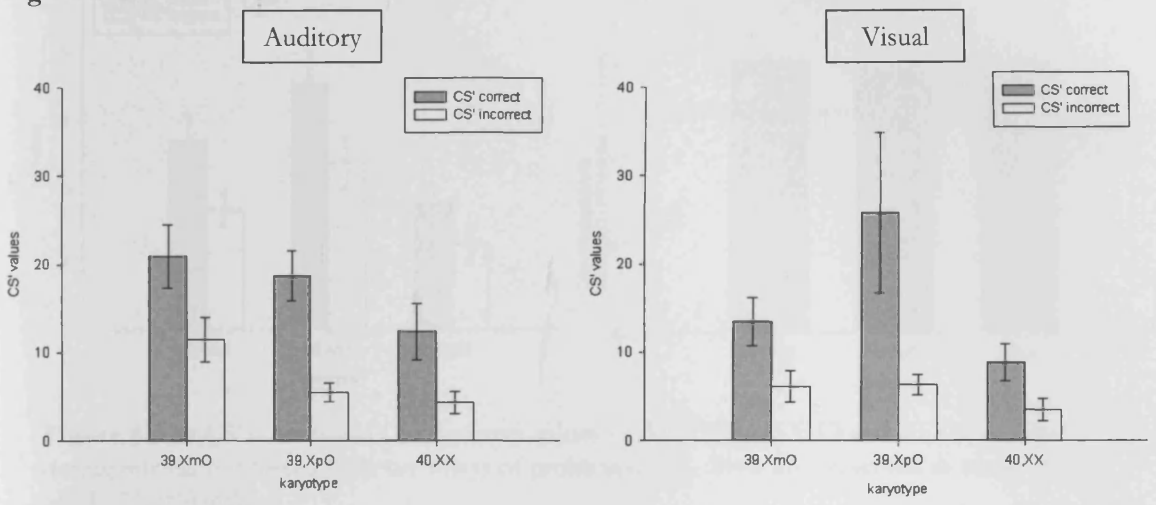


Figure 4.3.4d CS' correct and CS' incorrect for auditory and visual modalities on the last session of biconditional discrimination training, having achieved performance criterion on this session. Data are presented as mean values ± SEM.

4.3.5 Compound stimuli probe sessions

Probe sessions were a mixture of normal reinforced, and probe, trials. As described in detail earlier (see sections **4.2.4.1** and **4.2.4.5**), the four probe trials within the session consisted of two congruent and two incongruent trials which were either (1) tone & stimulus lights, (2) tone & house light, (3) buzz & stimulus lights and (4) buzz & house light. Whether these stimulus cue compounds were congruent or incongruent depended on the training contexts and the current contexts in which the animals were probed. Probe trials were not reinforced as uncontaminated responses were desired; this would prevent any influence on the animals' behaviour due to reinforcement, such as motivation and response side biases.

(i) Normal reinforced trials:

The performance in the normal reinforced trials was examined to ensure that during the probe sessions, animals were not overly disrupted by the presence of the probe trials and were still responding according to the stimulus-response associations that they had acquired during

biconditional discrimination training. Repeated Measures ANOVA with Within Subject factor RESPONSE (i.e. correct or incorrect CS' values) and Between Subject factor KARYOTYPE (i.e. comparison of 39,X^{m}O, 39,X^{p}O, 40,XX) was used to analyse the data.

Figure 4.3.5a

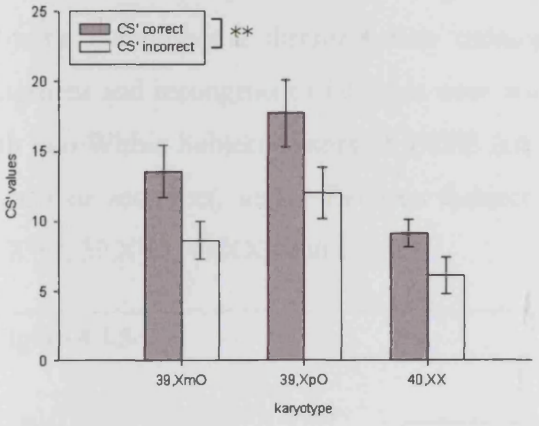


Figure 4.3.5b

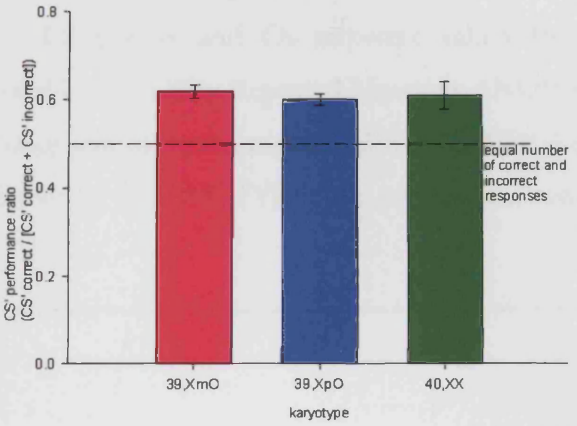


Figure 4.3.5a CS' correct and CS' incorrect values of 39,X^{m}O, 39,X^{p}O and 40,XX animals during normal reinforced trials on 8 days of probe sessions. Data are presented as mean values ± SEM. ** $p<0.001$.

Figure 4.3.5b Discrimination ratio of 39,X^{m}O, 39,X^{p}O and 40,XX animals from normal reinforced trials on 8 days of probe sessions. Ratios were calculated by CS' correct ÷ (CS' correct + CS' incorrect). Ratios over 0.5 would signify more correct than incorrect responses. Data are presented as mean values ± SEM.

Two out of seven 40,XX animals were excluded from the probe session analyses, as they showed a tendency towards increased CS' incorrect responding on normal reinforced trials rather than the expected tendency towards increased CS' correct responses averaged over the eight probe session days; this may be due to an effect of general task disruption during probe sessions, as these animals learned the initial discrimination to criterion. For the remaining 39,X^{m}O, 39,X^{p}O and 40,XX animals (**Figure 4.3.5a**), there were significantly more correct than incorrect RESPONSES ($F_{1,25} = 51.357$, $p<0.001$), and performance did not differ between KARYOTYPE groups ($F_{2,25} = 2.968$, n.s.). There was no significant RESPONSE × KARYOTYPE interaction ($F_{2,25} = 1.268$, n.s.). **Figure 4.3.5b** shows the discrimination ratios for the normal reinforced trials. As the previous analysis showed no karyotype differences, the data were grouped together and a One Sample t-test was conducted against the discrimination ratio value of 0.5 (i.e. chance level). The analysis showed that there was a significant difference between the performance of the animals and chance performance ($t_{27} = 11.073$, $p<0.001$). The results of the above analyses suggest that all karyotype groups were continuing to respond appropriately and above chance

level to the various stimulus-context compounds in the normal reinforced trials during probe sessions, and that subjects' performance were not significantly disrupted by the presence of probe trials.

(ii) Compound stimuli probe trials:

Probe trials were separated into congruent and incongruent types, based on the individual subject's biconditional discrimination training. CS correct and CS incorrect values from congruent and incongruent trial types were analysed by Two Way Repeated Measures ANOVA, with two Within Subject factors of TYPE (i.e. congruent or incongruent) and RESPONSE (i.e. correct or incorrect) and a Between Subject factor of KARYOTYPE (i.e. comparison with 39,X^{m}O, 39,X^{p}O, 40,XX, animals).

Figure 4.3.5c

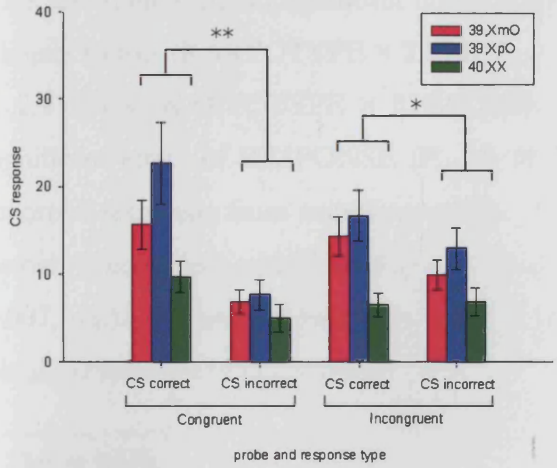


Figure 4.3.5c CS correct and CS incorrect values for congruent and incongruent trial types for 39,X^{m}O, 39,X^{p}O and 40,XX animals. Data are presented as mean values ± SEM. ** p<0.001, * p<0.01.

Figure 4.3.5d

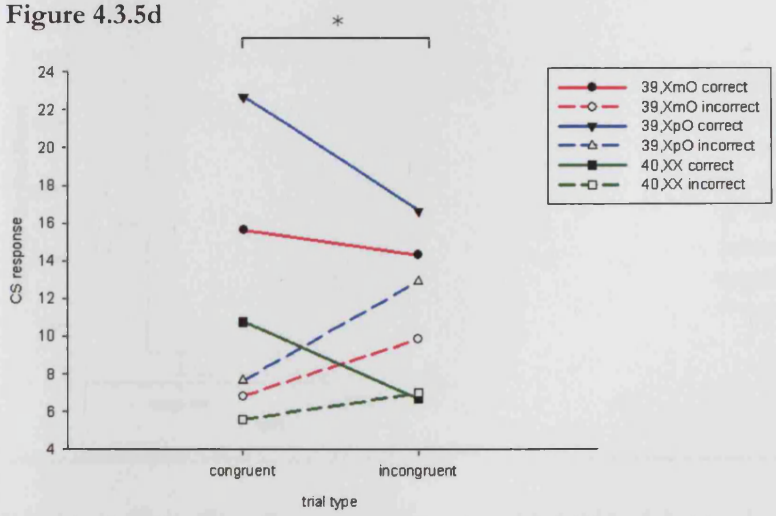


Figure 4.3.5d CS correct and CS incorrect values for congruent and incongruent trial types for 39,X^{m}O, 39,X^{p}O and 40,XX subjects, in a line graph for clarity. CSs correct and incorrect are represented by solid and dashed lines, respectively. * p<0.01, for the solid lines (CS correct) and for the dashed lines (CS incorrect).

For the 39,X^mO, 39,X^PO and 40,XX comparison (**Figure 4.3.5c**), most notably, there was a significant TYPE × RESPONSE interaction ($F_{1,25}$ = 21.341, $p<0.001$); *post hoc* tests revealed significantly higher CS correct than CS incorrect values in both congruent ($p<0.001$) and incongruent ($p<0.01$) trial types. The magnitude of the difference between CS correct and CS incorrect responses was larger in congruent trials than in incongruent trials (**Figure 4.3.5d**). This pattern of results suggests that subjects can perform both the congruent trials and the response conflict aspect (as indexed by incongruent trials) of this behavioural task successfully, but that, as expected, they had more difficulty with making a correct response when the responses dictated by the elements of the compound stimuli conflict i.e. in the incongruent trials. Additionally shown in the *post hoc*, CS correct values in congruent differed from that in incongruent trial types ($p<0.01$); this difference was also seen in CS incorrect values ($p<0.01$). This suggests that both correct and incorrect responses were significantly influenced by trial types. Animals performed similarly to each other, irrespective of KARYOTYPE ($F_{2,25}$ = 2.178, n.s.), and there were no significant interactions between KARYOTYPE and either/both Within Subject factors (KARYOTYPE × TYPE, $F_{2,25}$ = 0.479, n.s.; KARYOTYPE × RESPONSE, $F_{2,25}$ = 2.913, n.s.; KARYOTYPE × RESPONSE × TYPE, $F_{2,25}$ = 2.764, n.s.). There was also a significant effect of RESPONSE ($F_{1,25}$ = 29.791, $p<0.001$), which reflects more correct than incorrect responses from animals generally. Total CS values, irrespective of karyotype, summed across (1) congruent and (2) incongruent trials did not differ significantly (effect of TYPE, $F_{1,25}$ = 0.007, n.s.). **Figure 4.3.5e** shows a simple profile plot which explains the pattern of results obtained from the above analysis.

Figure 4.3.5e

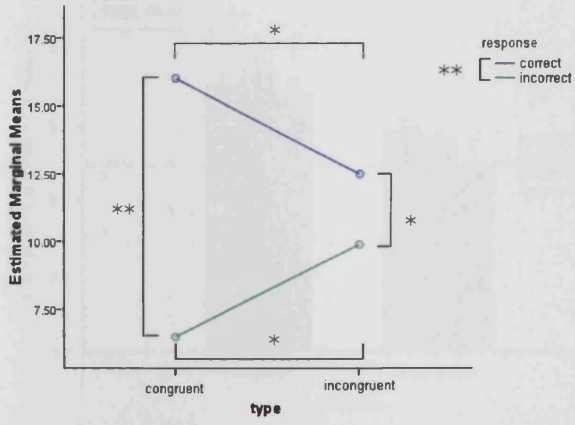


Figure 4.3.5e Estimated marginal means profile plot, showing significant differences in the analysis of the performance of 39,X^mO, 39,X^PO and 40,XX animals (karyotypes are grouped together).
** $p<0.001$, * $p<0.01$.

The above Two Way Repeated Measures ANOVA did not detect any KARYOTYPE effects, however, upon cursory inspection on **Figure 4.3.5c**, it would appear that 40,XX animals were

not able to make the correct response on incongruent trials (i.e. the CS correct and CS incorrect values were not different on incongruent trials). In the light of this, data were presented as discrimination ratios and re-analysed with a Repeated Measures ANOVA, with Within Subject factor of TYPE (i.e. congruent or incongruent) and Between Subject factor of KARYOTYPE (i.e. comparison of 39,X^mO, 39,X^pO and 40,XX animals). Similar pattern of results as the Two Way Repeated Measures was found; it was confirmed that there was a significant difference between the performance on congruent and incongruent trial types (main effect of TYPE, $F_{1,25}$ = 34.222, $p<0.001$), as animals were significantly better at resolving congruent than incongruent trials. Animals performed equivalently between KARYOTYPE groups ($F_{2,25}$ = 2.332, n.s.) and there was no significant interactions between KARYOTYPE and TYPE ($F_{2,25}$ = 0.778, n.s.). The data were then separated by karyotype groups and analysed by One-Sample t-tests, conducted against the discrimination ratio value of 0.5 (i.e. chance level; **Figure 4.3.5f**). For the congruent trial type, the discrimination ratios of all three karyotype groups were found to be significantly higher than chance level of 0.5 (39,X^mO: t_{12} = 7.930, $p<0.001$; 39,X^pO: t_9 9.699, $p<0.001$; 40,XX: t_4 = 5.435, $p<0.01$), suggesting animals were capable to perform on the congruent trials. For the incongruent trial type, the discrimination ratios of 39,X^mO (t_{12} = 3.905, $p<0.01$) and 39,X^pO (t_9 = 3.632, $p<0.01$) were significantly higher than chance level of 0.5, suggesting these animals were able to perform correctly in the trials with response conflict. However, for 40,XX animals, there were no significant differences between their discrimination ratio and chance level of 0.5 (t_4 = -0.044, n.s.); it would appear that 40,XX animals were performing at chance and were unable to respond correctly on the incongruent trials.

Figure 4.3.5f

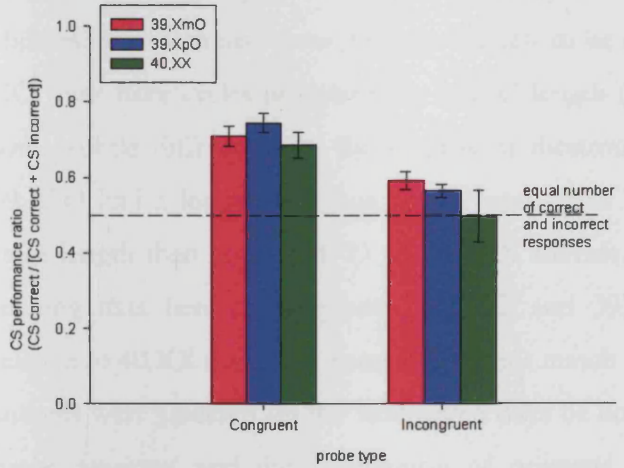


Figure 4.3.5f Discrimination ratios for congruent and incongruent trial types on probe sessions. Ratios were calculated by CS' correct ÷ (CS' correct + CS' incorrect). Ratios over 0.5 would signify more correct than incorrect responses. Data are presented as mean values ± SEM.

4.4 Discussion

In this experimental chapter, XO mice were tested on a novel murine biconditional discrimination task; the task required subjects to acquire two biconditional discriminations, one auditory and one visual, under two different contexts. After successful acquisition of the discriminations, animals were tested with congruent and incongruent audiovisual stimulus compounds. The present task examined executive function and response conflict; there is prior evidence that TS females are impaired in some aspects of executive function and response conflict (Ross *et al.*, 1995; Temple *et al.*, 1996; Kirk *et al.*, 2005), and display abnormal prefrontal cortex function (Tamm *et al.*, 2003). Additionally, a previous study with the XO mice (Davies *et al.*, 2005a), using a visual discrimination and reversal learning paradigm, has shown X-linked parent-of-origin effects on perseveration and re-acquisition of stimulus-reinforcer associations, and the orbitofrontal cortex, mediodorsal nucleus of the thalamus and hippocampus have been suggested to underlie these aspects of cognition.

The first main finding from this chapter was that both 39,$X^{m}O$ and 39,$X^{p}O$ (i.e. XO) mice learnt the two biconditional discriminations more slowly than 40,XX animals, as indexed by a higher number of sessions to performance criterion (i.e. an X-monosomy effect). There were no significant differences between the karyotype groups in their performance across the sessions in both auditory and visual modalities, which suggests that the slower learning in the XO animals was more likely to be a general impairment rather than due to dysfunction in one sensory domain. Importantly, XO mice showed an equivalent preference to XX mice for the reinforcers, suggesting they had similar motivation, and the karyotype groups performed equivalently in magazine and nose poke training, suggesting that the animals had the similar gross cognitive abilities. Furthermore, these data are unlikely to be confounded by oestrus status in that XX and XO mice have cycles of equivalent overall length (see Chapter III); in Chapter III, there were some subtle differences in the lengths of diestrous and oestrous stages of the cycle, in that 39,$X^{m}O$ had a longer diestrous stage length than 39,$X^{p}O$, and 39,$X^{p}O$ had a longer oestrous stage length than both 39,$X^{m}O$ and 40,XX animals. However, the biconditional discrimination training data here showed both 39,$X^{m}O$ and 39,$X^{p}O$ animals showing behavioural deficits relative to 40,XX mice; this pattern does not match that of oestrous stage lengths. Furthermore, animals were smeared on the first seven days of both biconditional discrimination learning and probe sessions, and the distribution of oestrous cycle stage for both time points was not different between karyotype groups (data not shown), suggesting that oestrous status is unlikely to confound the acquisition difference.

The second main finding was that while 39,X^{m}O and 39,X^{P}O animals were able to respond correctly on both congruent and incongruent trials in the probe sessions, 40,XX animals were only able to perform correctly on the congruent, but not incongruent, trials. All three karyotype groups showed a marked difference between correct and incorrect responses during congruent trials, and a smaller difference between correct and incorrect responses during incongruent trials (as they were more difficult due to response conflict), as expected. The oestrus status of mice was determined immediately following probe sessions daily; importantly, there were no gross effects of oestrus stage on performance on these probe trials. It was interesting to see that better performance from 39,XO mutant animals compared to 40,XX wildtype animals on incongruent trials (i.e. a 'beneficial' X-monosomy effect). Haddon & Killcross (2007) reported better performance on incongruent trials in rats with hippocampal formation lesions compared to sham operated rats; additionally, these hippocampal formation lesioned animals were sensitive to specific reinforcer devaluation on instrumental (lever press), but not on Pavlovian (magazine approach), measure of performance. It is possible that 39,XO mutant mice in my hands might have differences in the structure or function of hippocampal formation compared to 40,XX animals, caused by the haploinsufficiency of one or more X-linked genes that escapes X chromosome inactivation, and in future studies, it would be interesting to look into 39,XO animals' performance in specific reinforcer devaluation and their hippocampal function. It is worth noting that the subject number of 40,XX animals was low (n=5), and the low power of analysis will increase the probability of a Type II error; therefore, it is important to repeat the experiment with a higher number of subjects to ensure the difference in the performance between 39,XO and 40,XX animals on incongruent trials was indeed real.

Overall, these data suggest that there were no X-monosomy or X-linked parent-of-origin effects on brain processes underlying responses to congruent and incongruent stimuli. Whilst I can be relatively confident in excluding the possibility of X-linked parent-of-origin effect (since the performance of 39,X^{m}O and 39,X^{P}O animals was equivalent), with the low subject numbers of 40,XX mice used in the probe trials, it is not possible to discount completely the possibility of an X-monosomy effect which might not be apparent given low power of analysis. However, the fact that there was little variation within the 40,XX group and the small number of 40,XX mice tested performed equivalently to *Paf* heterozygotes (data not shown) does argue against an X-monosomy effect.

Acquisition of the biconditional discriminations requires the animal (i) to learn the stimulus-reward or stimulus-response associations (there is some debate as to which exact associative

structure is important here; Dickinson & De Wit, 2003) and (ii) to select the appropriate response given a particular stimulus (Adams *et al.*, 2001). Given that all animals could select the response appropriately during both the congruent and incongruent trials, this suggests that the slower acquisition observed in 39,$X^{m}O$ and 39,$X^{p}O$ mice is more likely to be a manifestation of impaired learning of the stimulus-reward or stimulus-reinforcer contingencies. There have not been any brain lesion studies in rats on this version of biconditional discrimination task that showed a deficit in the acquisition of the two biconditional discriminations, and therefore it is somewhat difficult to propose a candidate brain region which might underlie the current X-monosomy impairment. Part of the reason might be due to the different paradigms used in the rat and in the present study; with rats, a fixed number of sessions was given rather than testing the animals to performance criterion as in this experiment (Haddon & Killcross, 2005, 2006a, 2006b; Haddon *et al.*, 2008; Marquis *et al.*, 2007).

However, lesion studies in rats using conditional associative learning paradigms, i.e. tasks in which the acquisition of only one biconditional discrimination is required (stimulus A → nose poke left; stimulus B → nose poke right), have shown that a particular region of the striatum, the caudate nucleus, is important in the formation of stimulus-response associations, while the prefrontal cortex is thought to mediate mainly response selection (Winocur & Eskes, 1998; Adams *et al.*, 2001; Williams & Eskandar, 2006). Therefore, I might tentatively speculate that differences in the structure/function of the caudate nucleus might underlie differences in acquisition performance between 39,XO and 40,XX animals.

It is likely that there are one or more X-linked genes, which influence the development and/or ongoing function of brain regions mediating task acquisition (possibly including the caudate nucleus), and which escape X chromosome inactivation, such that the hypothetical gene dosage of these genes in 39,$X^{m}O$ and 39,$X^{p}O$ animals would be one, whereas that in 40,XX females would be two. To date, there are only a handful of X-linked genes, for example, *Eif2s3x, Jarid1c, Utx* and *Usp9x*, which are known to escape X chromosome inactivation in mice (Xu *et al.*, 2005, 2006, 2008a, 2008b; Brown and Greally, 2003; Yang *et al.*, 2010); however, to my knowledge, none of the known murine X-linked gene escapees has been shown to be expressed in the caudate nucleus. Follow up experiments could be conducted to examine whether specific lesions of the caudate nucleus could affect the acquisition of the two biconditional discriminations in this task. The 40,XY^{*X} mice, littermates from the 39,$X^{m}O$ generating cross, might be tested to examine if the behavioural effect on acquisition of the biconditional discriminations might be due to haploinsufficiency for one or more genes on the small Y^{*X} chromosome, which

encompasses the pseudoautosomal region and a small X-specific segment (Burgoyne *et al.*, 1998); a possible candidate on the Y^{*X} chromosome is the *Sts* gene which has been shown to modulate attentional functioning (Davies *et al.*, 2007).

Prior evidence from TS females suggests that these subjects are impaired with regard to the response conflict demands of the 'Stroop' task (Temple *et al.*, 1996; Kirk *et al.*, 2005); however, the present data did not show any impairment in the performance of the 39,XO mice during incongruent trials in which there was a conflict between two instrumental responses (in fact, 39,XO animals performed better than 40,XX animals on incongruent trials). Indeed, there have been instances where impaired performance during response conflict tasks in TS females has not been observed (Silbert *et al.*, 1977). This apparent species difference could be due to the presence of one or more X-linked genes in humans which influence the neurobiology underlying response conflict behaviour and escape X chromosome inactivation; the orthologous genes in mice might be autosomal, or might not escape X chromosome inactivation. As mentioned previously (see Chapter I), the murine X chromosome is thought to be much more extensively inactivated than its human counterpart (Goto & Monk, 1998; Lynn & Davies, 2007; Lopes *et al.*, 2010).

4.5 Summary

- There were no general behavioural differences between XX and XO with regard to training on the novel biconditional-response conflict task: (i) preference for both maltodextrin and sucrose over water increased significantly for every karyotype group across reinforcer preference test days, and there were no significant differences between karyotype groups in their preference of maltodextrin vs. sucrose, (ii) there were no significant differences between karyotype groups in the performance during magazine training, as indexed by number and duration of magazine entries, (iii) there were no significant differences between karyotype groups in the performance during nose poke training, as indexed by the number of sessions taken to reach each performance criterion at reinforcement schedule stages.

- There was a X-monosomy effect on the initial acquisition of the two biconditional discriminations whereby $39,X^{m}O$ and $39,X^{p}O$ animals required significantly more sessions to acquire the biconditional discriminations to the same performance criterion as 40,XX females.

- $39,X^{m}O$ and $39,X^{p}O$ animals were capable of completing both congruent and incongruent trials, but 40,XX animals could perform correctly only on congruent, but not incongruent,

trials. The difference between correct and incorrect responses was smaller in the incongruent (more difficult with response interference) than in the congruent trials, as expected.

Chapter V

Initial physiological and behavioural phenotyping of the Four Core Genotypes (FCG) mouse model

5.1 Introduction

In the General Introduction and previous experimental chapters, I considered, using the XO mouse model, two possible sex chromosome mechanisms (X-linked gene dosage and X-linked imprinting) which influence behaviour. In the rest of the experimental chapters to follow, I used a different mouse model in order to further investigate additional sex chromosome effects on behaviour; specifically I used the Four Core Genotypes (FCG) mouse model as a means of dissociating the brain and behavioural/gonadal hormonal effects of the Y-linked *Sry* gene from effects due to other sex-linked genes.

This chapter is an important pre-requisite to the other experimental chapters in the thesis that utilised the FCG animals for three reasons. First of all, although the model has been used previously by others in both behavioural (Gatewood *et al.*, 2006; Quinn *et al.*, 2007; McPhie-Lalmansingh *et al.*, 2008) and neurobiological (Carruth *et al.*, 2002; Wagner *et al.*, 2004) studies, there has been little systematic study of basic physiological and behavioural phenotypes associated with the various genetic manipulations used to alter *Sry* expression; moreover, some of these previous studies were carried out with the manipulations on a different genetic background (for example, Gatewood *et al.*, 2006 and McPhie-Lalmansingh *et al.*, 2008 used C57BL/6J whereas Quinn *et al.*, 2007 and De Vries *et al.*, 2002 used mice on MF1 background). Secondly, it is necessary to determine fundamental issues such as survivability and general health of the particular line of animals in my hands. Thirdly, it is important to be aware of any effects on basic sensory, emotional and motor processes that may confound interpretation of the data from the more complex behavioural analyses carried out later in the thesis.

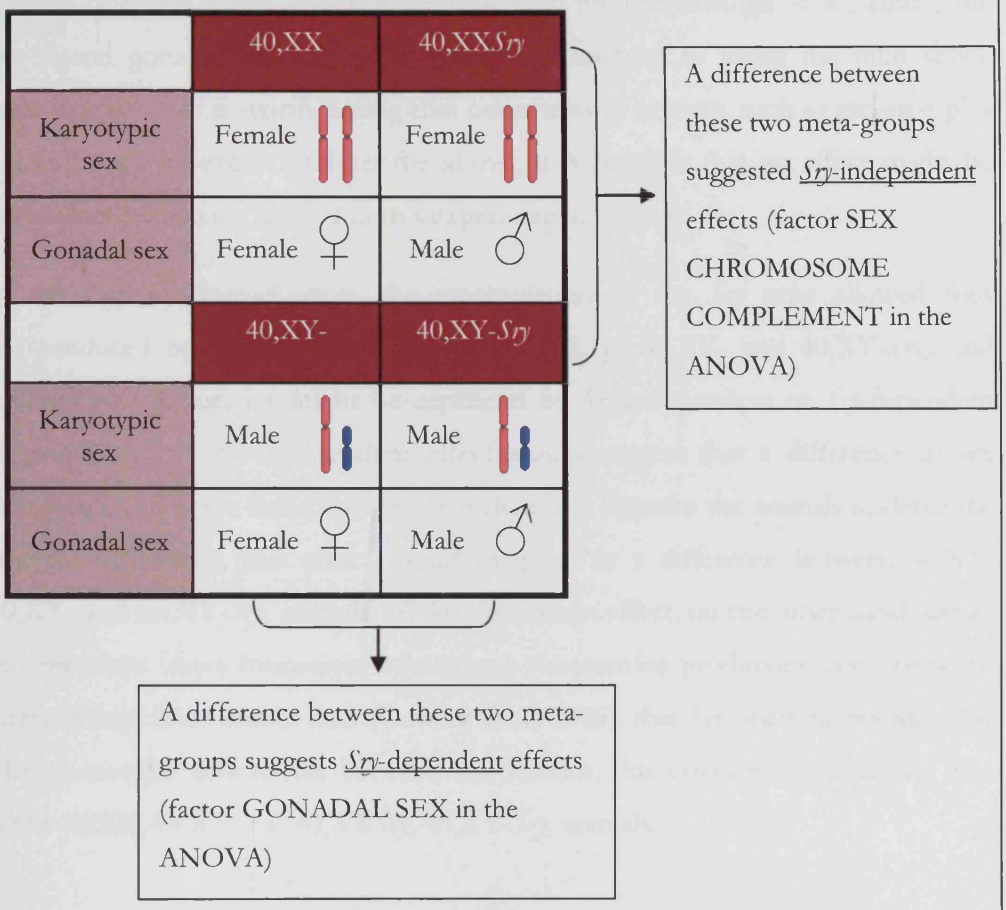


Figure 5.1a Diagram outlining the possible differences within the FCG mouse model and the associated effects, either *Sry*-independent or *Sry*-dependent. The statistical design of Two Way ANOVA with Between Subject factors of SEX CHROMOSOME COMPLEMENT (*Sry*-independent) and GONADAL SEX (*Sry*-dependent) was used to reveal any differences.

The behavioural battery of tests used is well established and similar to that performed with the XO mouse model (see Chapter III); briefly, physiological assessment of body weight and oestrous cycle was conducted, along with behavioural tests on the subjects' motor competence and stamina on the rotarod, activity in the locomotor activity box and fear reactivity on the elevated zero maze. Additionally, in this experimental chapter, testosterone hormone level in blood serum was measured; as the *Sry* gene has been manipulated in the FCG model and *Sry* plays a crucial role in testis determination, it was thought wise to investigate the level of testosterone. To my knowledge, published studies on the FCG mouse model have not observed any significant differences in the motor competence and stamina, or in locomotor activity, and therefore, I would not expect to find baseline behavioural differences in my subjects. There

have been reports, however, on possible *Sry*-dependent effects in anxiety, as indexed by performance on the one-way active shock avoidance (McPhie-Lalmansingh *et al.*, 2008); the researchers have found gonadal females to be quicker in learning to avoid the mild shock compared to gonadal males. It is worth noting that other tests of anxiety, such as elevated plus maze, did not detect such an effect. Given the above, it is possible that an effect might be observed in the elevated zero maze utilised in this experimental chapter.

As outlined in the General Introduction, the manipulation of the *Sry* gene allowed four genotypes to be produced by a single cross (40,XX, 40,XX*Sry*, 40,XY- and 40,XY-*Sry*), and differences between these genotypes might be explained by *Sry*-independent or *Sry*-dependent factors (see **Figure 5.1a**). A *Sry*-independent effect would suggest that a difference in sex chromosome complement (i.e. sex-linked genes other than *Sry*) between the animals underlie the observed phenotypic difference; this effect would manifest in a difference between 40,XX, 40,*XXSry* vs. 40,XY- and 40,*XY-Sry*, animals. A *Sry*-dependent effect, on the other hand, would suggest that *Sry*-dependent testes formation, subsequent testosterone production and hormonal variability, or alternatively, from recent data (Dewing *et al.*, 2006), that *Sry* brain expression, may contribute to the phenotypic differences between the animals; this effect would manifest in a difference between 40,XX, 40,XY- vs. 40,XX*Sry*, 40,XY-*Sry*, animals.

5.2 Materials and methods

5.2.1 Additional technical details concerning generation of FCG mice

An in-depth account of the generation of the FCG mice were given in Chapter I, and a general overview of how the FCG mice were generated and genotyped was provided in Chapter II. As noted, the FCG mice were generated by crossing an XY-*Sry* male with an XX female (**Figure 5.2.1a**). The Y- chromosome is a variant of Y^{129} derived from the mouse strain 129, deleted for the testis-determining gene *Sry* (*Tdy*^{m1} mutation, Lovell-Badge & Robertson, 1990). XY^{tdym1} males (referred to as XY-*Sry* males in this thesis) were originally generated on an MF1 background at MRC National Institute for Medical Research, UK in the laboratory of my collaborator Dr Paul Burgoyne (Mahadevaiah *et al.*, 1998), by introducing an *Sry* transgene derived from the transgenic line C57BL/6Ei-$Y^{AKR/J}$TgN(Sry-129)2Ei into pronuclear stage embryos from XY^{tdym1} females (referred to in this thesis as XY- mice); Y^{tdym1} is a 14kb deletion in the short arm of the Y chromosome which removes *Sry* (Lovell-Badge & Robertson, 1990; Gubbay et al., 1992). *Sry* is introduced as a fully penetrant transgene inserted onto an autosome; the location of the insertion and the copy number of the transgene is currently not known.

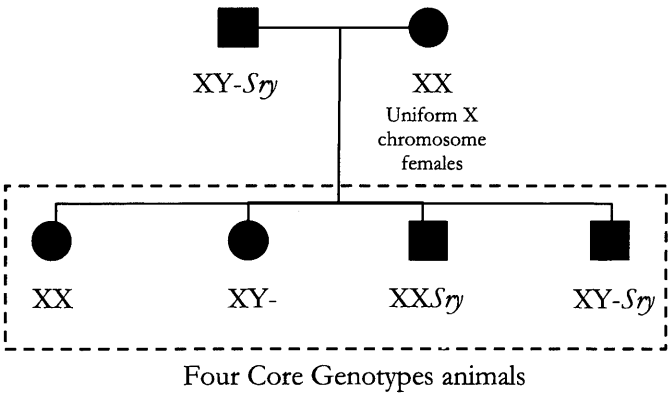


Figure 5.2.1a Details of the Four Core Genotypes generation cross. Black filled circles and squares denote gonadal females and males respectively.

By breeding the XY-*Sry* males with XX females with uniform X chromosome (for more information on the uniform X chromosome, please refer to the General Introduction), one can generate the four genotypes in the FCG cross; it was advantageous that the progeny of the four possible genotypes were produced in any given litter: 40,XX (gonadal females, karyotypic females), 40,XY- (gonadal females, karyotypic males), 40,XX*Sry* (gonadal males, karyotypic

females) and 40,XY-*Sry* (gonadal males, karyotypic males); hereafter referred to as XX, XY-, XX*Sry* and XY-*Sry* respectively, as shown in the simple breeding strategy diagram (**Figure 5.2.1a**). Subjects were first distinguished by external genitalia (gonadal sex), and then genotyped according to the methods described in Chapter II, **2.9.2.**

5.2.2 Subject numbers and animal husbandry

Testing was conducted when the majority of the FCG animals were aged from 6 to 12 months old. As blood testosterone level determination required trunk blood, the animals were culled after all necessary behavioural and cognitive testing was completed. **Table 5.2.2i** shows the number of subjects tested in the various tasks used in this chapter. General housing, handling and behavioural testing conditions were as described in Chapter II, **2.2** and **2.3.**

Table 5.2.2i Numbers of subjects (n) tested in the various physiological and behavioural tests.

Test	Genotype and 'n'			
	40,XX	40,XX*Sry*	40,XY-	40,XY-*Sry*
Body weight	22	15	12	18
Oestrus cycle length	17	N/A	10	N/A
Testosterone level	15	10	8	13
Rotarod	10	9	13	19
Locomotor activity	14	13	10	15
Elevated zero maze	13	11	9	13

5.2.3 Physiological assessments

5.2.3.1 Litter size, genotype distribution, mortality and general health

Litter size and mortality was determined by counting the number of offspring on postnatal day one, and by daily monitoring the number of dead or missing offspring on subsequent days until weaning (28 days). Offspring were tailed and genotyped, as described in Chapter II, **2.9.2.**, at age 35 days, and genotype distribution within litters was noted. General health of the subjects was monitored regularly throughout the testing period.

5.2.3.2 Body weight measurements

Stable body weights were determined when animals were six months old. Animals had ample time for habituation to housing environment and were accustomed to daily handling. Animals were weighed at the same time every day for five days. *Ad libitum* access to food and water was available.

5.2.3.3 Oestrous cycle

Vaginal smearing was performed regularly during the initial handling and at intervals during the experiment.

5.2.3.4 Testosterone levels determination

Testosterone levels were assayed from the blood serum of adult mice, as described in Chapter II, **2.10**.

5.2.4 Behavioural assessments

5.2.4.1 Rotarod

Subjects were tested on the rotarod apparatus (described in Chapter II, **2.7.1**) to test their motor function and balance under conditions of accelerating and constant rotation speed. Experimental procedures are as described in Chapter III, **3.2.4.1**.

5.2.4.2 Locomotor activity

Movements in, exploration of, and habituation to a novel environment by subjects were tested in the locomotor activity boxes, described in Chapter II, **2.7.2**. Experimental procedures are as described in Chapter III, **3.2.4.2**.

5.2.4.3 Elevated zero maze

Fear reactivity was assessed using the elevated zero maze, described in Chapter II, **2.7.3**. Experimental procedures are as described in Chapter III, **3.2.4.3**.

5.2.5 Statistical analyses

Data were analysed using SPSS software (version 17, SPSS Inc., IBM, U.S.A.). Data were subject to Two Way ANOVA with Between Subject factors GONADAL SEX (i.e. the presence or absence of testis indexing the presence or absence of *Sry*) and SEX CHROMOSOME

COMPLEMENT (i.e. karyotype of the animal being either XX or XY), in order to identify any *Sry*- dependent or independent effects. Additional factors specific to particular analyses related to the various tests are defined in the relevant Results section below and were, where repeated measures used, analysed by SPANOVA[16]. Additionally, Greenhouse-Geisser (epsilon of 0.75 or lower) or Huynh-Feldt (epsilon of 0.75 or higher) corrections were applied to degrees of freedom if the Mauchly's Test of Sphericity was violated in Repeated Measure tests. Data with covariate factors were subject to ANCOVA. When initial ANOVA revealed a significant effect, Tukey HSD Test was performed for *post hoc* comparisons, and when a significant interaction was revealed, Least Significant Difference adjustment was used for *post hoc* pairwise comparisons. Chi-square test for goodness of fit was carried out on non-parametric data. For all comparisons, p values of <0.05 were regarded as significant.

[16] SPANOVA is a Repeated Measures ANOVA with a two way mixed split-plot design, in which one independent variable is repeated measures and the other independent variable consists of independent groups.

5.3 Results

5.3.1 Physiological data

5.3.1.1 General health, litter size, mortality and genotype distribution

Close monitoring of the FCG animals did not indicate any particular general health problems in any of the four genotypes. In this experimental cohort, a total of 16 litters and 109 pups were born, with an average litter size of 6.8 (**Table 5.3.1.1i**); pre-weaning mortality was low (2.75%), with all but three pups surviving up to weaning. The three pups that died came from the same litter. There was no difference in genotype distribution in the remaining 106 offspring (χ^2 (3, N = 106) = 3.057, n.s.). Mortality of adult animals was low (<5%) and did not differ between genotype groups.

Table 5.3.1.1i Number of litters and pups surviving up to postnatal day 28 (average age of weaning), mortality and genotype distribution of FCG animals. Numbers in brackets refer to the total number of litters and pups born, including those that subsequently died before weaning.

Number of litters	15 (16)			
Number of pups	106 (109)			
Postnatal mortality	2.75%			
Mean litter size	6.8			
Genotype distribution				
	XX	XX*Sry*	XY-	XY-*Sry*
Number of pups	26	34	23	23

5.3.1.2 Body weight

Average body weights from adult animals measured over five days were significantly higher in the gonadal males (XX*Sry* and XY-*Sry*) than gonadal females (XX and XY-; **Figure 5.3.1.2a**; main effect of GONADAL SEX, $F_{1,63}$ = 189.262, p<0.001). There was no effect of sex chromosome complement on weight (XX*Sry*, XX vs. XY-*Sry*, XY-; effect of SEX CHROMOSOME COMPLEMENT, $F_{1,63}$ = 0.115, n.s), and no interaction between the two factors ($F_{1,63}$ = 1.482, n.s). Health was closely monitored over the testing period and it was unlikely that any underlying health problems could contribute to this weight variability.

Figure 5.3.1.2a

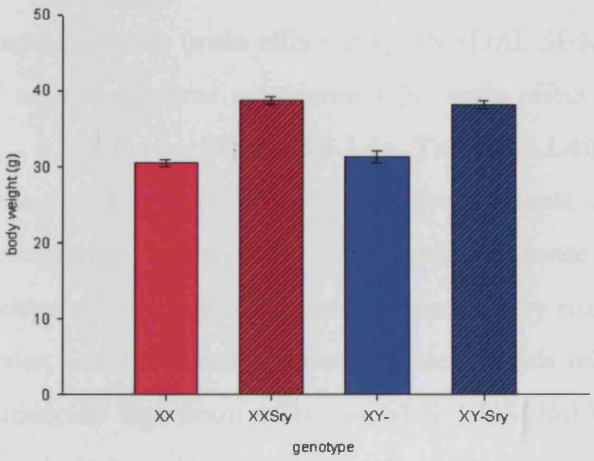


Figure 5.3.1.2a The baseline body weight of FCG mice at 6 months old, with *ad libitum* food and water. Data are presented as mean values with ± SEM.

5.3.1.3 Oestrous cycle

Repeated Measures ANOVA was used to analyse the data on oestrous cycle in gonadal females, with Between Subject factor of GENOTYPE and Repeated Measure factor STAGE (diestrous, proestrous, oestrous). The length of each cycle stage in days was not significantly different between XX and XY- females (effect of GENOTYPE, $F_{1,25} = 0.989$, n.s.). There was in addition no significant interaction between GENOTYPE and STAGE ($F_{1.593,39.827} = 0.497$, n.s.). As expected, there was a main effect of STAGE ($F_{1.593,39.827} = 15.035$, $p < 0.001$) reflecting the well established mouse oestrous cycle, with a significantly shorter proestrous stage compared to diestrous and oestrous stages, which was the case in all animals irrespective of genotype (**Figure 5.3.1.3a**).

Figure 5.3.1.3a

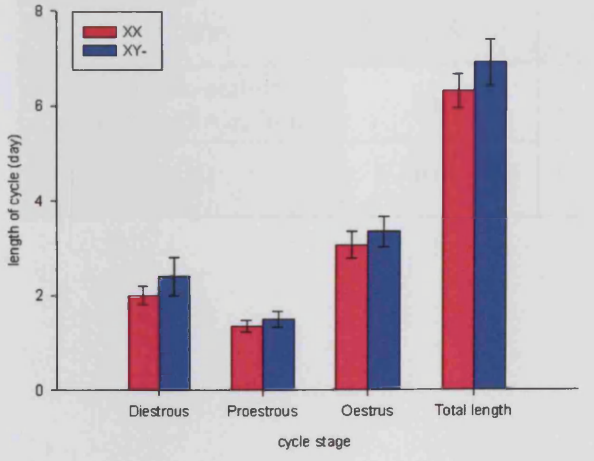


Figure 5.3.1.3a Total length of oestrous cycle and length at each stage (diestrous, proestrous and oestrous) as determined by vaginal smears from female mice aged 6 months. Data are presented as mean values with ± SEM.

5.3.1.4 Serum testosterone levels determination

As expected, gonadal males were found to have significantly higher levels of testosterone than gonadal females (main effect of GONADAL SEX, $F_{1,42} = 5.766$, $p < 0.05$); this was irrespective of sex chromosome complement (no main effect of SEX CHROMOSOME COMPLEMENT, $F_{1,42} = 2.876$, n.s.; **Figure 5.3.1.4a**, **Table 5.3.1.4i**). No interaction between the two factors was observed ($F_{1,42} = 0.677$, n.s.). However, simple observation of the data seems to indicate that testosterone levels were modulated to some extent by sex chromosome complement independently of gonadal sex (in that XX*Sry* males showed reduced levels relative to XY-*Sry* males, and XX females showed reduced levels relative to XY- females), although there was no statistically significant effect of SEX CHROMOSOME COMPLEMENT (see above). This issue is discussed further at the end of the chapter.

Figure 5.3.1.4a

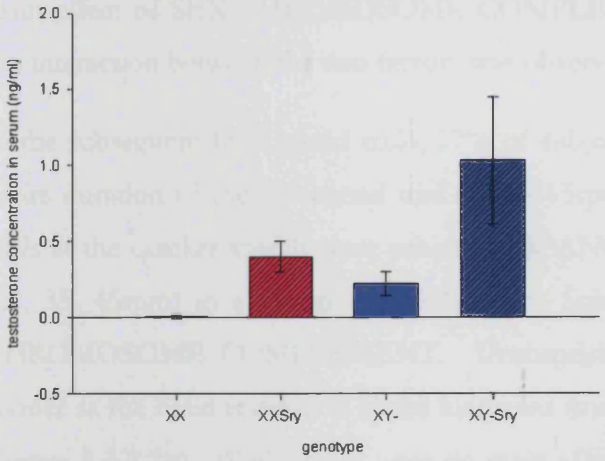


Figure 5.3.1.4a Blood serum hormone levels (ng/ml) as determined by ELISA. Testosterone concentration values were calculated using a standard curve from known concentration standard solutions and the optical density obtained from samples. Data are presented as mean values ± SEM (see also **Table 6.3.1.4i**).

Table 5.3.1.4i Mean ±SEM values for trunk blood testosterone levels for the four genotype groups (BLD: below limits of detection; assay sensitivity: 0.083ng/ml).

Genotype	XX	XX*Sry*	XY-	XY-*Sry*
Mean testosterone concentration (ng/ml)	BLD	0.400	0.224	1.031
±SEM	BLD	0.098	0.078	0.418

5.3.2 Behavioural data

5.3.2.1 Reactivity to handling

During the initial handling of the subjects, all animals showed the typical mild signs of distress, which included urination and faecal deposition. After three days of handling, these signs had subsided in most animals. There did not appear to be an obvious effect of genotype on the degree of initial distress and habituation. Vaginal smearing led to some signs of distress in females, including urination and faecal deposition, but again, most signs subsided after three days of habituation to the procedure.

5.3.2.2 Rotarod

Rotarod was used to assay motoric competence. All subjects performed equivalently during the accelerating rotation speed trials (no main effect of GONADAL SEX, $F_{1,47}$ = 0.018, n.s.; no main effect of SEX CHROMOSOME COMPLEMENT, $F_{1,47}$ = 1.494, n.s., **Figure 5.3.2.2a**). No interaction between the two factors was observed ($F_{1,47}$ =0.054, n.s.).

In the subsequent fixed speed trials, 77% of subjects were able to stay on the apparatus for the entire duration of the 60 second trial at low 15rpm constant revolution speed. Data from the trials at the quicker speeds were subject to SPANOVA, with Repeated Measure factor SPEED (25, 35, 45rpm) in addition to the Between Subject factors of GONADAL SEX and SEX CHROMOSOME COMPLEMENT. Unsurprisingly, the animals tended to fall off the bar sooner as the fixed revolution speed increased (main effect of SPEED, $F_{2,94}$ = 32.030, $p<0.001$, **Figure 5.3.2.2b**). Whilst there were no main effects for Between Subject factors (GONADAL SEX, $F_{1,47}$ = 3.634, n.s.; SEX CHROMOSOME COMPLEMENT, $F_{1,47}$ = 0.018, n.s.), there was a significant interaction between SPEED and GONADAL SEX ($F_{2,94}$ = 3.801, $p<0.05$). *Post hoc* tests showed that gonadal females (XX and XY-) performed significantly better than gonadal male (XX*Sry* and XY-*Sry*) subjects on the faster 35rpm and 45rpm revolution speed blocks ($p<0.05$), irrespective of sex chromosome complement. Gonadal females had been shown to be significantly lighter in weight as adults than gonadal males (**Figure 5.3.1.2a**); hence this effect may have been something to do with being more agile, in general, on the balancing bar.

Figure 5.3.2.2a

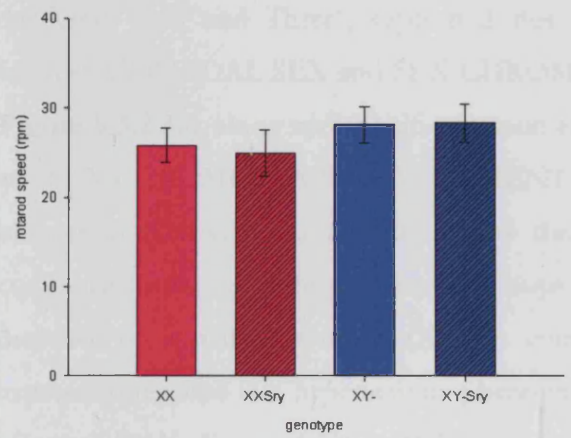


Figure 5.3.2.2b

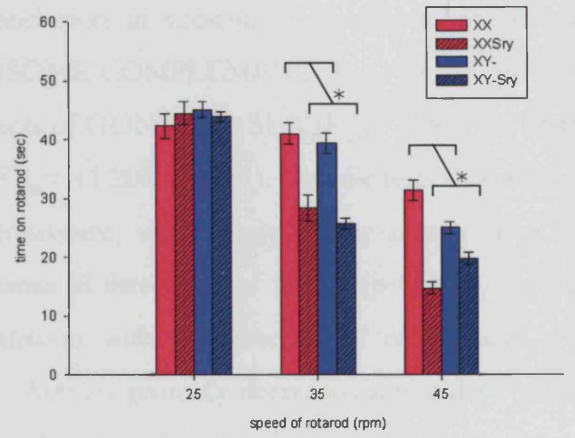


Figure 5.3.2.2a The speed at which subjects fell off the rotarod apparatus in the two probe trials with accelerating rotation speed. Data are presented as mean values ± SEM.

Figure 5.3.2.2b Time spent on the rotarod in the four fixed rotation speed trial blocks. Data from 15rpm were not shown. Data are presented as mean values ± SEM. * $p<0.05$

Whilst this data could imply that *Sry* influences fine motor performance, it is also possible that body weight could influence performance given that I found that gonadal males tended to be heavier than their gonadal female counterparts. Data from my laboratory have shown that smaller mice tend to perform better on this task than larger mice, irrespective of their genotype (e.g. Relkovic, Ph.D thesis, 2009). In the light of this, data analysis (Repeated Measures ANOVA with Within Subject factor of SPEED [25, 35 and 45 rpm] and Between Subject factors of GONADAL SEX and SEX CHROMOSOME COMPLEMENT) was repeated with an additional covariate of weight; this analysis suggested that when weight has been taken into account, there was no effect of SPEED ($F_{2,92} = 0.108$, n.s.), and no effect of GONADAL SEX ($F_{1,46} = 0.314$, n.s.) and SEX CHROMOSOME COMPLEMENT ($F_{1,46} = 0.021$, n.s.). Therefore, the superior performance of gonadal females at higher speeds on the rotarod is likely to be mainly due to their smaller size relative to gonadal males; indeed, during testing the experimenter observed that the females (especially those lighter in weight) tended to grip onto the bar and rotate with it rather than walking on the bar.

5.3.2.3 Locomotor activity

(i) Mean breaks across three testing sessions:

There were effects on locomotor activity in that the XX, XX*Sry*, and XY-*Sry* animals showed similar activity in terms of mean number of breaks per two hour session over the three days of

testing, whilst XY- mice were consistently and significantly more active than the other three genotype groups. Statistical analysis using SPANOVA, with Repeated Measures factor DAY (i.e. day One, Two and Three), supported this conclusion in showing a significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,48} = 7.835$, $p<0.01$; **Figure 5.3.2.3a**), along with significant main effects of GONADAL SEX ($F_{1,48} = 7.843$, $p<0.01$) and SEX CHROMOSOME COMPLEMENT ($F_{1,48} = 11.208$, $p<0.01$). *Post hoc* tests showed the interaction observed was contributed by the consistent, significantly higher activity of XY- compared to the other three genotype groups across all three days of testing ($p<0.001$), i.e. the presence of a male karyotype (XY) in combination with the absence of endogenous *Sry* expression resulted in a hyperactivity phenotype. Activity generally decreased across days (main effect of DAY, $F_{2,96} = 4.508$, $p<0.05$), as expected from habituation to a novel environment. Furthermore, there was no significant interaction between DAY and GONADAL SEX ($F_{2,96} = 0.673$, n.s.) and between DAY and SEX CHROMOSOME COMPLEMENT ($F_{2,96} = 0.966$, n.s.), which suggests that all groups habituated to the novel environment at comparable rates across the three days.

Figure 5.3.2.3a

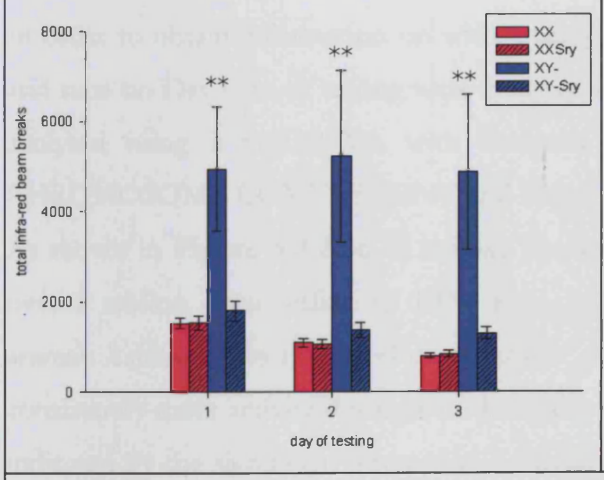


Figure 5.3.2.3b

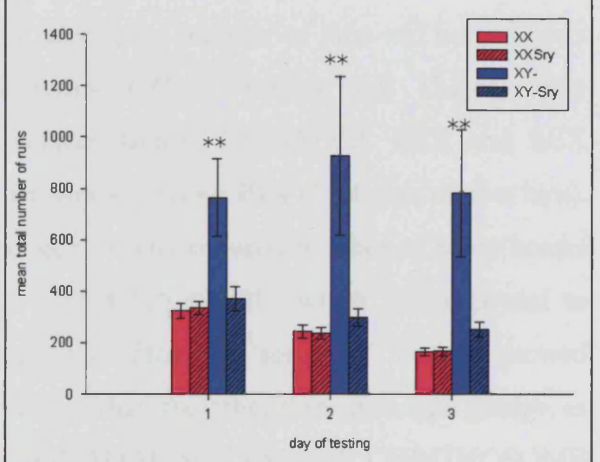


Figure 5.3.2.3a Locomotor activity, as indexed by the mean number of single infra-red beam breaks per two hour session, over three consecutive days of testing. Data are presented as mean values ± SEM. ** $p<0.001$

Figure 5.3.2.3b Locomotor activity, as indexed by the mean number of runs per two hour session, over three consecutive days of testing. Data are presented as mean values ± SEM. ** $p<0.001$

(ii) Mean runs (consecutive beam breaks, one after the other, which signified the animal running across the cage) across three testing sessions:

The data with mean number of runs per two hour session over three days (**Figure 5.3.2.3b**) were also analysed with SPANOVA with the aforementioned factors. A similar qualitative pattern of results were obtained with a significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,48} = 8.205$, $p<0.01$), and significant main effects of GONADAL SEX ($F_{1,48} = 8.069$, $p<0.01$) and SEX CHROMOSOME COMPLEMENT ($F_{1,48} = 12.489$, $p<0.01$). As before, *post hoc* tests showed the interaction observed was due to the consistent, significantly higher activity of XY- compared to the other three genotype groups across all three days of testing ($p<0.001$), i.e. the presence of a male karyotype (XY) in combination with the absence of endogenous *Sry* expression resulted in a hyperactivity phenotype in both the breaks and run data. Again, in general, activity decreased across days (main effect of DAY, $F_{1.712,82.179} = 6.486$, $p<0.01$), with no significant interaction between DAY and GONADAL SEX ($F_{1.712,82.179} = 2.604$, n.s.) and between DAY and SEX CHROMOSOME COMPLEMENT ($F_{1.712,82.179} = 1.966$, n.s.).

(iii) Five-minute bin data over Day One:

In order to obtain information on within-session effects, the number of infra-red beam breaks and runs on Day One of testing were analysed in terms of 24, five minute bins. The data were analysed using a SPANOVA with Between Subject factors GONADAL SEX and SEX CHROMOSOME COMPLEMENT and Repeated Measure factor BIN (1-24, five minute bins). As shown in **Figure 5.3.2.3c** all animals showed significantly reduced number of beam breaks over a session (main effect of BIN, $F_{5.512,264.584} = 55.396$, $p<0.001$), which was expected as animals habituated to the novel environment over time. However, again XY- animals showed consistently more activity throughout the entire session than the other three genotype groups, as indicated by the significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,48} = 8.073$, $p<0.01$) and additionally, by significant main effects of GONADAL SEX ($F_{1,48} = 7.995$, $p<0.01$) and SEX CHROMOSOME COMPLEMENT ($F_{1,48} = 11.074$, $p<0.01$); these data, reflecting within-session behaviours, confirmed the previous mean data obtained across sessions. A similar pattern of effects was seen in the runs data, with again the XY- mice showing increased activity within the session (GONADAL SEX × SEX CHROMOSOME COMPLEMENT, $F_{1,48} = 9.114$, $p<0.01$; **Figure 5.3.2.3d**).

Figure 5.3.2.3c

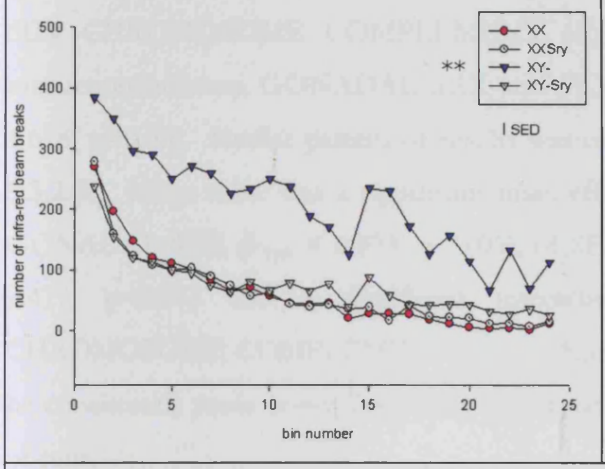


Figure 5.3.2.3d

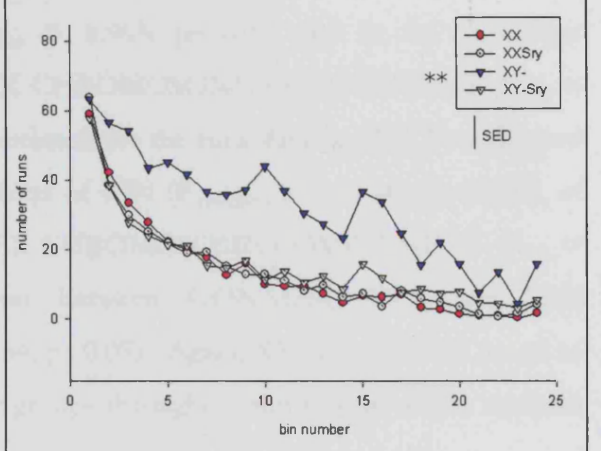


Figure 5.3.2.3c Number of infra-red beam breaks over the session of Day One of testing. The session was separated into 24 bins of five minutes each. Data are presented as mean values. ** $p<0.001$

Figure 5.3.2.3d Number of runs over the session of Day One of testing. The session was separated into 24 bins of five minutes each. Data are presented as mean values. ** $p<0.001$

The analyses from beam break and run data also revealed that there was a significant difference in the rate of habituation across the session between XY- and the other three genotype groups. With beam breaks, the interaction between the factors BIN and GONADAL SEX was found to be significant ($F_{5.512,264.584} = 2.682$, $p<0.05$), while the interaction between BIN and SEX CHROMOSOME COMPLEMENT was not ($F_{5.512,264.584} = 1.827$, n.s.). With runs, both interactions were found to be significant (BIN × GONADAL SEX, $F_{7.183,344.787} = 2.186$, $p<0.05$; BIN × SEX CHROMOSOME, $F_{7.183,344.787} = 2.24$, $p<0.05$). These data suggest that the activity of XY- animals at the beginning of the session was comparable to that of the other groups; however, XY- animals habituated at a slower rate throughout the session and took longer to reach the activity plateau near the end of the session than the other groups.

(iv) Five-minute bin data over Days Two and Three:

Given that there was a significant effect in Day One, five-minute bin data over Days Two and Three were analysed to see if the behavioural pattern from the XY- animals continued throughout testing. Data were analysed as described above in Part (iii). As shown in **Figure 5.3.2.3e**, all animals showed significantly reduced number of beam breaks over the session on Day Two (main effect of BIN, $F_{4.430,212.655} = 22.508$, $p<0.001$), which reflected habituation to the test environment over time. As with the five-minute bin data on Day One, XY- animals were

found to be consistently more active than other genotype groups throughout the entire session; this was reflected in significant main effects in GONADAL SEX ($F_{1,48}$ = 6.939, $p<0.05$) and SEX CHROMOSOME COMPLEMENT ($F_{1,48}$ = 8.963, $p<0.01$), and in the significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,48}$ = 6.618, $p<0.05$). Similar pattern of results was obtained for the runs data on Day Two (**Figure 5.3.2.3f**), where there was a significant main effects of BIN ($F_{5.548,266.285}$ = 36.846, $p<0.001$), of GONADAL SEX ($F_{1,48}$ = 6.873, $p<0.05$), of SEX CHROMOSOME COMPLEMENT ($F_{1,48}$ = 9.477, $p<0.01$) and a significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,48}$ = 6.554, $p<0.05$). Again, XY- animals were found to be consistently more active than other genotype groups throughout the session on the measure of runs.

Figure 5.3.2.3e

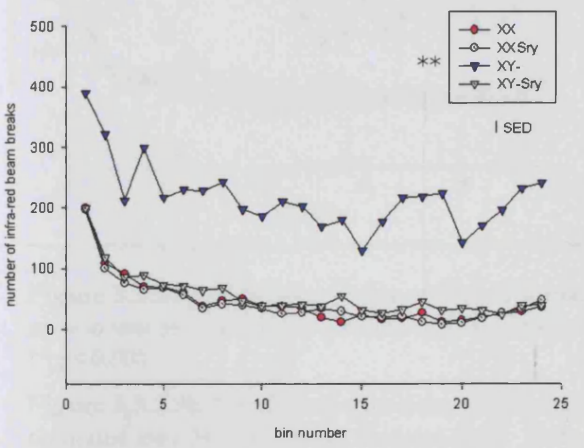


Figure 5.3.2.3f

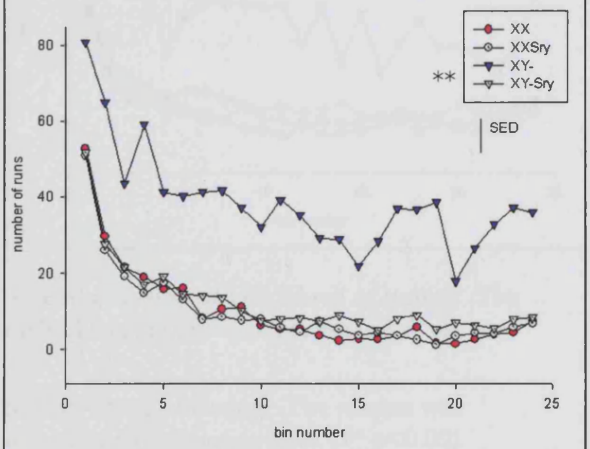


Figure 5.3.2.3e Number of infra-red beam breaks over the session of Day Two of testing. The session was separated into 24 bins of five minutes each. Data are presented as mean values. ** $p<0.001$

Figure 5.3.2.3f Number of runs over the session of Day Two of testing. The session was separated into 24 bins of five minutes each. Data are presented as mean values. ** $p<0.001$

The same analyses were conducted for data from Day Three. As previously observed on other test days, all animals habituated to the testing environment over the session (**Figure 5.3.2.3g**), which was reflected by the significant main effect of BIN in beam breaks ($F_{4.645,222.984}$ = 10.211, $p<0.001$). In terms of beam breaks, XY- animals were once again found to be more active than other genotype groups over the entire session; this was shown in the significant main effects of GONADAL SEX ($F_{1,48}$ = 7.080, $p<0.05$) and of SEX CHROMOSOME COMPLEMENT ($F_{1,48}$ = 11.535, $p<0.01$), and in the significant interaction between GONADAL SEX and SEX

CHROMOSOME COMPLEMENT ($F_{1,48}$ = 7.363, p<0.01). Similar pattern of results was obtained from the analysis on the runs data on Day Three (**Figure 5.3.2.3h**); there was a significant main effects of BIN ($F_{3.681,176.664}$ = 18.825, p<0.001), of GONADAL SEX ($F_{1,48}$ = 7.326, p<0.01), of SEX CHROMOSOME COMPLEMENT ($F_{1,48}$ = 12.966, p<0.01) and a significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,48}$ = 7.485, p<0.01). In terms of runs, XY- animals were found to be consistently more active than other genotype groups throughout the session.

Figure 5.3.2.3g

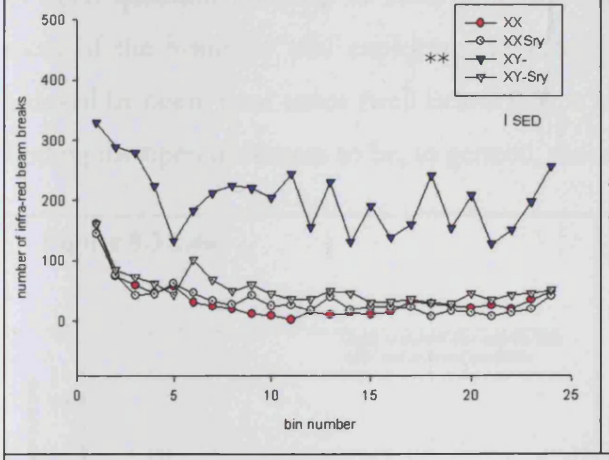


Figure 5.3.2.3h

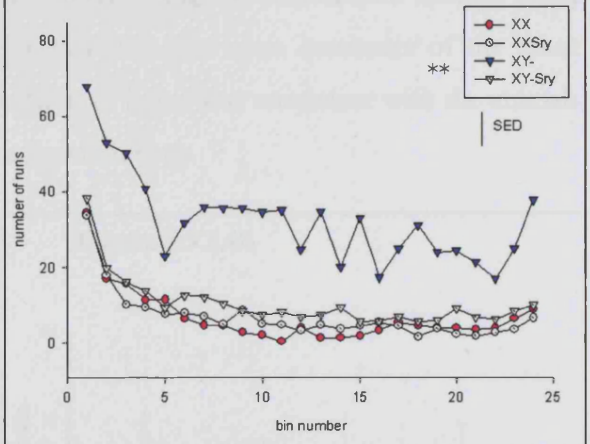


Figure 5.3.2.3g Number of infra-red beam breaks over the session of Day Three of testing. The session was separated into 24 bins of five minutes each. Data are presented as mean values. ** p<0.001

Figure 5.3.2.3h Number of runs over the session of Day Three of testing. The session was separated into 24 bins of five minutes each. Data are presented as mean values. ** p<0.001

It is worth noting that the significant difference in the rate of habituation across the session observed on Day One was not similarly found in Day Two and Three. In contrast, on Day Two and Three, on both measures of beam breaks and runs, there were no interactions between BIN and GONADAL SEX[17], and between BIN and SEX CHROMOSOME COMPLEMENT[18]. In other words, on Day Two and Three, the XY- animals were more active than the other genotype groups throughout the session and there were no differences in the rate of habituation, i.e. at the

[17] Beam breaks on Day Two: $F_{4.43,212.655}$ = 0.823, n.s.; runs on Day Two: $F_{5.548,266.285}$ = 1.236, n.s.; beam breaks on Day Three: $F_{4.645,222.984}$ = 1.247, n.s.; runs on Day Three: $F_{3.681,176.664}$ =1.044, n.s..

[18] Beam breaks on Day Two: $F_{4.43,212.655}$ = 0.985, n.s.; runs on Day Two: $F_{5.548,266.285}$ = 0.725, n.s.; beam breaks on Day Three: $F_{4.645,222.984}$ = 1.429, n.s.; runs on Day Three: $F_{3.681,176.664}$ =1.231, n.s..

beginning of the session, the XY- animals were already more active and this continued throughout, *cf.* Day One, in which XY- animals were comparable in activity to other groups at the beginning of the session and took longer to reach the activity plateau at the end of the session. This pattern of behaviour suggests that additional to XY- animals being slower to habituate to a novel environment, these animals also displayed a higher basal rate of activity in an environment which was no longer completely novel.

5.3.2.4 Elevated zero maze

The time spent in the open and enclosed parts of the elevated zero maze was calculated as ratios of open quadrant duration to total duration. As shown in **Figure 5.3.2.4a**, all animals spent most of the 5-minute trial exploring the closed rather than the open quadrants of the maze indexed by open/total ratios (well below 0.5), a pattern of behaviour consistent with the animals finding the open quadrants to be, in general, more fear-inducing.

Figure 5.3.2.4a

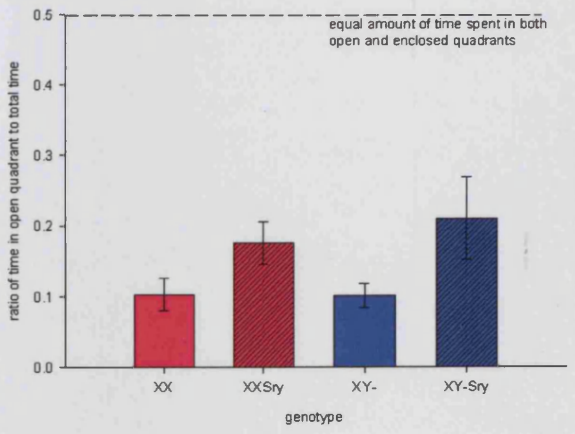


Figure 5.3.2.4b

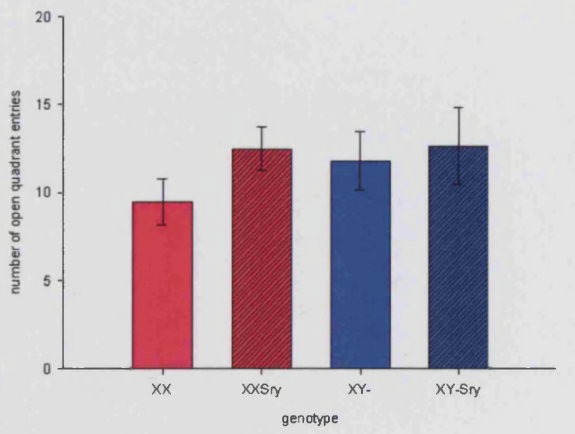


Figure 5.3.2.4a Ratio of duration spent exploring open quadrant to total time in the maze. Data are presented as mean values ± SEM.

Figure 5.3.2.4b Number of entries into the open quadrant. Data are presented as mean values ± SEM.

Gonadal male (XX*Sry* and XY-*Sry*) animals spent significantly more time in the open quadrants than gonadal females (XX and XY-), irrespective of sex chromosome complement, as indicated by a main effect of GONADAL SEX ($F_{1,42}$ = 5.336, $p<0.05$) and no main effect of SEX CHROMOSOME COMPLEMENT ($F_{1,42}$ = 0.177, n.s.; **Figure 5.3.2.4a**). There was also no interaction between the two factors ($F_{1,42}$ = 0.210, n.s.). The number of open quadrant entries (**Figure 5.3.2.4b**) did not differ between genotype groups; there were no main effects of

GONADAL SEX ($F_{1,42}$ = 1.265, n.s.), nor SEX CHROMOSOME COMPLEMENT ($F_{1,42}$ = 0.529, n.s.), nor GONADAL SEX × SEX CHROMOSOME COMPLEMENT interaction ($F_{1,42}$ = 0.401, n.s.).

5.4 Discussion

In this experimental chapter, I examined the basic physiological and behavioural phenotypes associated with the FCG model, using a standard battery of tests (body weight measurements, oestrous cycle determination, blood serum testosterone level assessment and behavioural assays of motoric function, locomotor activity and anxiety). This was necessary for several reasons: to replicate and extend previous and limited data obtained using this model on a different genetic background, to examine survivability and general health of the mice from the FCG model in my hands, and to be aware of any potential confounds on sensory, motivational, emotional and motor processes relevant to subsequent cognitive tests.

I found there was an equal distribution of the four genotypes of the FCG model, as ascertained at weaning, which suggests that there was no disproportionate incidence of *in utero* or early postnatal mortality in any one group. XY-*Sry* and XX mice from the cross bred readily, producing litters of equivalent size to wildtype MF1 mice, and all four groups exhibited low rates of mortality. The vast majority of FCG mice appeared healthy with no overt physical impairments up to the age of 24 months, when they were culled. Gonadal males were found to be significantly heavier in body weight than gonadal females at age of six months. The two groups of female mice (XX and XY-) displayed similar oestrus cycle stage lengths at six months of age. Blood serum testosterone levels of gonadal males were significantly higher than that of gonadal females, which was not surprising as testosterone is secreted primarily from Leydig cells in the testis (Wu *et al.*, 2007). However, it would appear that blood serum testosterone levels were modulated to a degree by sex chromosome complement independently of gonadal sex, such that XY-*Sry* had more testosterone than XX*Sry* males, and testosterone levels of XY- females were higher than that of XX females (although this pattern of results did not reach significance), suggesting that the presence of the Y- chromosome (with the absence of *Sry* gene) is associated with a trend towards higher testosterone levels. Y chromosomes from different mouse strains have been shown to influence serum testosterone levels (Selmanoff *et al.*, 1977), and the mouse Y chromosome carries ten distinct genes with open reading frames that are likely to be functional, with roles in testis function (Toure *et al.*, 2004).

The most striking result arising from the behavioural work in this chapter was an effect on locomotor activity, whereby XY- females habituated to the novel environment (i.e. Day One) at a slower rate and were significantly hyperactive (as shown in data on Day Two and Three), compared to the other three genotype groups. This observation is consistent with previous data comparing activity in the FCG model in an open field test, which also showed that XY- mice

were the most active of the four genotype groups, although this was not significant (McPhie-Lalmansingh *et al.*, 2008). Together these findings suggest that, only in the presence of a male XY karyotype, the lack of *Sry* results in increased levels of activity and slower habituation to the novel environment. To explain this complex pattern of results, I speculate that there is some, as yet unidentified, interaction between *Sry* and one or more genes on the Y chromosome which might modulate locomotor activity and/or habituation.

A second main finding in this chapter was that gonadal males (XX*Sry* and XY-*Sry*) spent more time exploring the open quadrants of the elevated zero maze than gonadal females (XX and XY-), which implies that the former group is less anxious than the latter. Importantly, as both groups exhibited similar numbers of quadrant entries, this result cannot be due to activity effects. Recent work by McPhie-Lalmansingh and colleagues (2008) using the FCG model also showed an effect of gonadal type on the latency to escape a mild shock on the one-way active avoidance test, but there was no gonadal effect on behavioural measures on the elevated plus maze; both one-way active avoidance and elevated plus maze are tests of anxiety (Handley and McBlane, 1993). This apparent discrepancy between the present study and the data from elevated plus maze in the McPhie-Lalmansingh *et al.* study may be explained by subtle differences between the behavioural tasks used (as detailed in the Discussion section in Chapter III), between test conditions, by the different strains used (MF1 in the present study vs. C57BL/6J), or by the differing ages of the subjects (around 12 months in the present study vs. 4 months). However, the finding presented here has since been replicated within my laboratory using the same elevated zero maze task and a different group of FCG mice (Eleni Kopsida, pers. comm.), and thus appears to be a robust effect. The data suggest that *Sry* (independently of other genes on the sex chromosomes) reduces the component of anxiety indexed by the elevated zero maze.

It was also found that whilst performance on the accelerating rotarod was not influenced by genotype (consistent with previous data obtained by McPhie-Lalmansingh *et al.*, 2008), performance on the higher speed trials of the rotarod (highly motorically demanding) was influenced by gonadal sex; specifically, gonadal males fell off the apparatus more quickly than gonadal females. However, subsequent data analysis, which included a covariate of weight, showed that the heavier body mass of gonadal males contributed to their poorer performance on.

Sry-dependent effects on body weight and on the elevated zero maze could be mediated via two possible mechanisms: by Sry acting directly on the brain, or through Sry influencing gonadal hormone secretion indirectly through its effects on testis differentiation. It was shown that, as expected, blood serum testosterone levels were higher in mice with a *Sry* transgene than in mice

without. These testosterone data from the present study are similar to that obtained by others in the FCG model (Gatewood *et al.*, 2006). Hence, the above physiological and behavioural effects could have been mediated by the effects of testosterone; indeed there is evidence that testosterone increases muscle size in normal males (Bhasin *et al.*, 1996) and in hypogonadal men (Bhasin *et al.*, 1997), and provides anxiolytic effects as indexed by the elevated plus maze in rodents (Frye *et al.*, 2008a; Frye *et al.*, 2008b). From the present data, I might expect wildtype (XY) males to be less anxious than wildtype females as indexed by the elevated zero maze; however, published data on sex differences in animal models of anxiety have been variable (Johnston & File, 1991; Rodgers & Cole, 1993; Palanza, 2001) and these studies were conducted with the elevated plus maze rather than the elevated zero maze as in the present study. Additionally, the difference in body weight between the gonadal males and gonadal females might reflect motivational differences in their general feeding behaviours; however, this could not be verified as their day-to-day feeding patterns were not monitored.

In summary, this chapter has shown that the FCG model is amenable to sophisticated behavioural and cognitive testing, but that potential effects on activity, anxiety and body weight (influencing motor competence) should be taken into consideration in future analyses. Compared to other published studies, genetic background and testing in different laboratories do not seem to have major effect on survivability, breeding and health of the FCG mice, although there may be subtle effects on some aspects of behaviour.

5.5 Summary

- The four genotypes from the FCG were generated in equal proportion. FCG mice exhibit good breeding performance and low levels of mortality.
- Gonadal males were found to be heavier than gonadal females at six months of age.
- There was no difference in oestrus cycle in the gonadal females from the FCG model at six months of age.
- As expected, gonadal males were found to have higher blood serum testosterone levels than gonadal females.
- Gonadal males were impaired on the most motorically demanding trials of the rotarod compared to gonadal females; this effect is probably due to the larger size of the former group.

- XY- mice were found to be more active than the other three genotypes in a locomotor activity test. The higher activity was observed on all days of testing.
- Gonadal males spent more time exploring the open quadrants of the elevated zero maze than gonadal females, possibly reflecting a lower level of anxiety in the former group.
- Differences in body weight and elevated zero maze performance between gonadal males and gonadal females are likely to be due to group differences in testosterone levels.

Chapter VI

Simple visual discrimination and reversal learning in Four Core Genotypes (FCG) mouse model

6.1 Introduction

Sry can theoretically affect brain development and function in two ways: via direct effects on the brain, or via effects on gonadal hormone secretion (notably testosterone, in either an organizational or activational manner). **Table 6.1i** illustrates how testosterone may influence a range of behaviours, especially cognitive behaviours, in man, primates and rodents.

Table 6.1i A selection of the many studies in man, primates and rodents which show the influence of testosterone on a range of cognitive behaviours

Species	**Author(s)**	**Main findings**
Man	Young *et al.*, 2010	Large changes in testosterone levels did not affect cognition. There was an association between increasing testosterone level and better mental rotation task performance.
Man	Eisenegger *et al.*, 2010	Single dose of testosterone administered sublingually in women increased fair bargaining behaviour and social interaction efficiency.
Man	Möller *et al.*, 2010	Testosterone added to oestrogen treatment in menopausal women impaired immediate verbal memory compared to oestrogen treatment alone.
Man	Matousek & Sherwin, 2010	Significant curvilinear relationship between working memory and testosterone level was found in older men.
Primate	Morris *et al.*, 2009	Gonadectomy increases prepulse inhibition in postpubertal rhesus macques.
Primate	Hagger & Bachevalier, 1991	Female infant rhesus monkeys learnt a concurrent visual discrimination task quicker than males. Female androgenisation led to performance similar to normal infant males, while male gonadectomy resulted in performance similar to normal females.

Rat	Guillamon *et al.*, 1986	In a T-maze discrimination task, there was no sex difference between normal males and females on acquisition of the task. Females learnt the reversed contingency quicker than males; this effect was reversed with female androgenisation and male gonadectomy.
Rat	Kritzer *et al.*, 2001	Gonadectomy impairs T-maze acquisition in adult male rats.
Rat	Sandstrom *et al.*, 2006	Gonadectomy impairs working memory retention at longer, but not shorter, retention intervals in a spatial memory task in male rats.
Mice	Grgurevic *et al.*, 2008	SF-1 KO mice are without gonads and adrenal glands. In the absence of sex steroids replacement, adult SF-1 KO mice (both sexes) were more aggressive towards stimulus females. Following sex steroid replacement, control males were more aggressive towards intruders than control females, SF-1 KO males and SF-KO females.
Mice	Frye *et al.*, 2008a	Aged, intact male mice, when given testosterone metabolites, increased anti-anxiety and antidepressant behaviours in open field, light-dark transition, mirror maze and forced swim tasks. Testosterone metabolites also increased anti-anxiety behaviours in elevated plus maze, elevated zero maze and the Vogel task, and increased motor behaviour, latency to fall on the rotarod and cognitive performance on hippocampally-mediated, but not amygdale-mediated, portion of the conditioned fear and inhibitory avoidance tasks. Similar patterns were observed in young, gonadectomised male mice.
Mice	Frye *et al.*, 2008b	Gonadectomised male mice, when given testosterone metabolites, displayed reduced anxiety on elevated plus maze and increased performance on object recognition task.
Mice	Benice & Raber, 2009	Spatial memory retention in the water maze was improved by testosterone treatment in aged female mice.

Table 6.1i has shown that testosterone plays an important role in shaping many different behaviours and cognition, ranging from prepulse inhibition, anxiety, memory, and more interestingly in terms of this thesis, learning in visual discrimination and reversal tasks. In addition, as Hagger & Bachevalier (1991) and Guillamon *et al.* (1986) demonstrated, testosterone works independently of sex chromosome complement. Most studies have shown the activational effects of testosterone (for example, through gonadectomy); however, the study by Grgurevic *et al.* (2008), with the use of the SF-1 KO mice, investigated the organisational effect of testosterone (since these SF-1 KO mice were never exposed to testosterone).

In contrast, data on the influences of sex chromosome mechanisms (see Chapter I, **1.2.3.1**) on physiology and behaviour are rather sparse. Much of these data have come from rodent models. Dewing *et al.* (2006) found that *Sry* gene expression in the rat brain, independent of gonadal hormones, had a direct effect on behaviour, with downregulation of *Sry* in the substantia nigra in male rats leading to motor deficits. In terms of neurobiology, the number of dopamine neurons

in rodent mesencephalon or diencephalon (Carruth *et al.*, 2002) and density of vasopressin-immunoreactive fibres in the lateral septum (De Vries *et al.*, 2002), was found to be linked with sex chromosome complement. With regard to behaviour, sex chromosome complement was found to affect aggression (Gatewood *et al.*, 2006; Canastar *et al.*, 2008), social interactions such as grooming and sniffing (McPhie-Lalmansingh *et al.*, 2008) and nociception (Gioiosa *et al.*, 2008a, 2008b) in mice. The extent to which cognition can be influenced by sex chromosome mechanisms remains relatively unexplored; Quinn *et al.* (2007) found sex chromosome complement regulated habit formation in a food-reinforced instrumental paradigm and Barker *et al.* (2010) showed that XY animals formed a response habit for alcohol quicker than XX subjects, irrespective of gonadal sex. Further insights into the role of sex chromosome effects on physiology, behaviour and cognition in man might come from studying sex chromosome disorders such as Turner syndrome (TS) and Klinefelter's syndrome (KS); for example, short stature in TS has been linked to SHOX gene deficiency (Ross *et al.*, 2001) and neurocognitive profile in TS girls (e.g. impaired visuospatial abilities) has been associated with distal Xp22.3 (Ross *et al.*, 2000; Zinn *et al.*, 2007a). Impaired fear recognition in TS has been linked with *EFHC2* gene locus (Weiss *et al.*, 2007), although this has not been replicated in another study (Zinn *et al.*, 2007b). With regard to Klinefelter's syndrome, 12 genes on the X chromosome, which were differentially expressed in KS males, were found to correlate significantly with verbal cognitive abilities (Vawter *et al.*, 2007); additionally, it was suggested that the parental origin of the extra X chromosome inherited was linked with motor and language problems (Stemkens *et al.*, 2006).

A seminal study by Skuse *et al* (1997) in TS subjects showed an X-linked parent-of-origin effect on behavioural inhibition; specifically, TS subjects inheriting their single X chromosome from their mother (45,X^m) exhibited longer inhibition latencies in the Same-Opposite World task than subjects who inherited their single X chromosome from their father (45,X^p). The same study also showed that normal males (who inherit their single X chromosome from their mother, i.e. 46,X^mY^p) were impaired on the same psychological test relative to females (who inherit one X chromosome from each parent, i.e. 46,X^mX^p). The authors suggested that these data could be explained by the presence of one or more imprinted genes on the X chromosome. Subsequent work using the 39,XO mouse model (modelling TS in humans), undertaken in this laboratory, showed that on a behavioural task which was thought to tax similar psychology to that of the Same-Opposite World task (reversal learning), 39,X^mO mice were impaired on the main measure of behavioural inhibition relative to 39,X^pO mice, recapitulating the pattern of human data. A novel X-linked imprinted gene *Xlr3b* was discovered which was hypothesised to be responsible

for this behavioural effect (Davies *et al.*, 2005a). As X-linked imprinted genes may be expressed differently in males and females (in both man and mouse; see Chapter I), the above behavioural results may be regarded as being due to sex chromosome complement effects.

In reversal learning tasks, a subject must first acquire two parallel stimulus-reinforcer associations (e.g. stimulus A equals reinforcer, stimulus B equals no reinforcer), which are then subsequently reversed, such that new and opposite associations must be learned with inhibition of the old associations. Reversal learning tasks have been well established and conducted with humans, primates and rodents. In monkeys, Dias and colleagues (1996) have found damage to the lateral prefrontal cortex and to orbitofrontal cortex leads to inhibitory control loss in attention and in affective processing respectively. For humans, Overman (2004) has found sex differences in object reversal performance in young children, and Yuan and colleagues (2008), using an ERP design, have found gender differences in behavioural control in adult humans. In rats, there is some evidence that males and females acquire a simple visual discrimination at the same rate, but that upon reversal, females were quicker to learn the new stimulus-reinforcer contingency (Guillamon *et al.*, 1986). The authors speculated that this difference was primarily mediated by gonadal hormones as female androgenisation/male gonadectomy, on day one after birth, reversed the pattern of results.

Hence, it is clear that there is evidence that behavioural inhibition across species may be influenced by gonadal hormones and/or sex chromosome complement. The purpose of this chapter is to exploit the Four Core Genotypes (FCG) model to determine the extent and specificity with which gonadal hormones and sex chromosome complement contribute to the acquisition of stimulus-reinforcer associations and to behavioural inhibition processes using a simple two-way visual discrimination with reversal task. The reversal learning task employed in this study was similar to that used previously (Davies *et al.*, 2005a), and is well established within the laboratory. Briefly, a Y-maze design was used, which was set up for a simple two-way non-spatial visual discrimination with two goal arms (one black, and one white), with one coloured arm consistently baited with a liquid reinforcer; the position of the two arms was pseudorandomly switched to prevent the mice from using spatial strategies to solve the task. Subjects were required to learn the stimulus-reinforcer contingency to a high degree of accuracy; once reaching criterion of over 85% correct for three consecutive sessions, this stimulus-reinforcer contingency was reversed so that the previously unreinforced arm was then baited. The number of errors committed in reaching a high level of accuracy on this reversed stimulus-reinforcer contingency provided an index of subjects' ability to inhibit pre-potent responses and

to acquire novel stimulus-reinforcer contingencies (see Methods below). This test allows multiple, dissociable measures of initial learning, asymptotic performance, reversal of the learning contingencies and subsequent learning of the new contingencies to asymptotic performance.

As detailed in the General Introduction and Chapter V, the FCG mouse model produces four different genotypes: 40,XX, 40,XX*Sry*, 40,XY- and 40,XY-*Sry*. Differences between these animals might be explained by *Sry*-independent (i.e. sex linked genes other than *Sry*) and *Sry*-dependent (i.e. gonadal hormones and/or direct *Sry* effects in the brain) effects. Given the large number of published studies on the effects of gonadal hormones on cognition (including reversal learning), and the comparatively fewer number of findings on the role of sex chromosome complement in behaviours, I would expect any observed effects more likely to be *Sry*-dependent rather than *Sry*-independent.

In addition to the FCG mouse model, wildtype XY animals were also tested in this task. All published studies to date, with the exception of De Vries *et al.* (2002), have tested and analysed the FCG mouse model as a whole without additional controls (using a Two Way ANOVA design, see **Section 6.2.6** for more details). However, I wished to test wildtype XY animals alongside the FCG mouse model to investigate the extent to which the XY-*Sry* males were equivalent to wildtype XY animals, and whether any differences exist between the XY-*Sry* and wildtype XY in a cognitive task. De Vries *et al.* (2002) had conducted behavioural and neurobiological studies using both FCG mouse model and wildtype XY animals, and had observed some interesting differences between XY-*Sry* and wildtype XY males, for example, differences in masculine sexual behaviour between XY-*Sry* and wildtype XY males, and lower number of TH-ir neurons in the anteroventral periventricular nucleus of the preoptic region in transgenic *Sry* animals (XX*Sry* and XY-*Sry*), compared to gonadal FCG females (XX and XY-) and wildtype XY males. Note that no cognitive tasks were used in the De Vries study.

6.2 Materials and methods

6.2.1 Subjects and animal husbandry

Animals from the Four Core Genotypes (FCG) mouse model (XX, XY-, XX*Sry*, XY-*Sry*) were subject to behavioural testing. Subjects were aged six months at the start of testing. Details on general animal husbandry and handling can be found in Chapter II (**2.2**, and **2.3**), and generation cross particulars can be found in Chapter II (**2.1.1**) and V (**5.2.1**). The number of subjects tested in the simple discrimination and reversal learning task is summarised in **Table 6.2.1i**.

Table 6.2.1i. Numbers of subjects (n) used in statistical analyses for the three main phases of the task. The table also includes the initial arm colour used in the acquisition phase. Numbers in parentheses referred to the total number of animals tested, including those subsequently excluded (see **6.2.6** for exclusion criteria).

Phase of testing	Genotype and 'n'			
	40,XX	40,XX*Sry*	40,XY-	40,XY-*Sry*
Reinforcer preference test	17 (18)	11	8 (10)	14 (15)
Acquisition phase	17 (18)	11	9 (10)	15
Reversal/re-acquisition phase	17 (18)	11	9 (10)	15
Colour of arm	Initial reinforced maze arm in acquisition phase			
black	9	8	4 (5)	5
white	8 (9)	3	5	10

Wildtype XY male mice used in this thesis were generated by crossing XY MF1 males (Harlan, U.K.) with uniform X chromosome females (see Chapter I, for information on uniform X chromosome); this was to ensure the X chromosome in the wildtype XY male progeny was the same as that in the FCG mice and therefore reducing variability between the two groups of subjects. Information on general animal husbandry and handling can be found in Chapter II (**2.2**, and **2.3**). Eleven wildtype XY male mice were tested in parallel with the FCG mice.

6.2.2 Body weight and oestrus status

Throughout the experiment the body weights of subjects were monitored regularly and oestrus status of female subjects was determined by vaginal smearing. Smearing was performed immediately after testing, on the first seven days of both acquisition and reversal/re-acquisition phases. Details of the procedure can be found in Chapter II, **2.4**.

6.2.3 Reinforcer preference test

Subjects were placed on the water restriction schedule two weeks prior to reinforcer preference test (Chapter II, **2.5**). The habituation and preference for the reinforcer (10% condensed milk solution, Nestlé, U.K.) were assessed. General details of this procedure can be found in Chapter II, **2.6**. When presented with both water and reinforcer after some exposure, animals usually displayed a clear preference for the latter. However, if animals did not at all sample the reinforcer during the 6-day testing, they were given a 'forced' sampling session on the seventh day, in which both containers held the reinforcer in order to force them to sample the milk. On the following day, these animals were subject to a session of normal preference testing (one water container, one reinforcer container), and the reinforcer preference was recorded.

6.2.4 Simple visual discrimination and reversal learning task

Upon completion of the reinforcer preference testing, training in the simple visual discrimination and reversal learning task began and the apparatus used is described in Chapter II, **2.7.5**. Throughout the testing period the mice were maintained on the routine water restriction schedule in order to motivate performance in the maze.

6.2.4.1 Habituation to the apparatus

Animals were given a 10-minute habituation session in the apparatus. Subjects were placed in the transparent start arm and were free to explore the maze without reinforcement. This habituation session was to familiarise the subjects to the maze apparatus and to detect any underlying bias in behaviour, for example, a strong preference for a particular arm colour. As described in Chapter II, **2.7.5**, infra-red beam breaks were recorded to give information on the number of entries into, and the amount of time spent in, each of the two goal arms.

6.2.4.2 Acquisition phase

Subjects were given a 10-trial session per day until criterion performance was achieved (see below). For each subject, a black or white goal arm was pseudorandomly assigned as the

'reinforcer' arm (detailed in **Table 6.2.1i**), which food well contained the reinforcer (30µl of 10% condensed milk solution). Note that the reinforcer could not be seen from choice space located at the centre of the maze. At the beginning of each trial, each subject was placed in the transparent start arm, breaking the 280mm infra-red beam and opening all guillotine doors. As the subject moved into the central choice space and broke the 20mm infra-red beams at one of the two goal arms, the guillotine door to the start arm would automatically drop to prevent return. A time limit of two minutes was implemented, after which, the subject was gently nudged into the central choice space and the door to the start arm was manually shut. The subject was then required to make a 'choice' (see later, section **6.2.5**) between the two goal arms; there was no time limit for this choice and no intervention was allowed. Finally, the guillotine door of the chosen arm to the central choice space was closed when the animal reached the end of the chosen arm, broke the 280mm infra-red beam and consumed the reinforcer (if any), after which the trial ended and the animal was removed to a holding box until the next trial. During the inter-trial interval (ITI; 55 seconds), the maze was thoroughly cleaned with 1% acetic acid solution and the reinforcer was placed once again in the reinforcer arm. The position of the reinforcer arm was pseudorandomly switched between trials to prevent formation of spatially based strategies, but the colour of the reinforcer arm remained unchanged throughout the acquisition phase. After the ITI, the animal was re-introduced into the maze at the start arm and the next trial began. The acquisition phase continued until the animal achieved an average of 85% correct per day over three consecutive days (defined as stable, performance criterion). The total number of incorrect choices (see section **6.2.5**) made to reach acquisition criterion was recorded.

6.2.4.3 Probe session to test odour cue usage

By thoroughly cleaning the goal arms with 1% acetic acid after each trial, this should ensure that no odour cue strategies were used to achieve performance criterion. To confirm that this was the case, a probe session was performed once the stable performance criterion was reached. During this probe session, in trials 2, 4, 6, 8 and 10, two completely new, but in all other respects identical, goal arms were used to assess usage of odour cues, since performance would be predicted to be disrupted (to chance levels) by this task manipulation if odour cues were guiding choice behaviour.

6.2.4.4 Reversal of task contingency

After the olfactory cue probe session, subjects were required to re-achieve performance ≥90% correct on a single session in order to progress to the reversal phase of the task. The stimulus-reinforcer contingency was reversed, such that the reinforcer arm in the reversal phase was switched to that previously not reinforced in the acquisition phase; i.e. subjects previously reinforced in the black arm were now reinforced in the white arm and *vice versa*. The subject was required to learn this new stimulus-reinforcer contingency, as before, to the re-acquisition performance criterion which was an average of 85% correct per day over three consecutive days. The total number of incorrect choices made to reach re-acquisition performance criterion was recorded and separated into 'below-chance' 'perseverative errors' (where the subject continued to choose the once correct but now incorrect goal arm) and 'above-chance' errors (which indexed the learning of the new contingency) (see section **6.2.5**).

6.2.5 Definition of behavioural measures

Figure 6.2.5a shows details of the maze infra-red beam placements used to define the behavioural measures. A choice was defined as the breakage of the 70mm (one body length) infra-red beam in one of the goal arms. After a choice has been registered, the guillotine door to the arm not chosen would close automatically. A correct choice was defined as breakage of the 70mm infra-red beam in the reinforced goal arm and incorrect choice was the breakage of the infra-red beam in the non-reinforced arm. The following response latencies were recorded. Start latency was defined as the time elapsed between the breakage of the 280mm infra-red beam in the start arm and the breakage of the 20mm infra-red beam in the start arm. Choice latency was defined as the time elapsed between the breakage of the 20mm infra-red beam in the start arm and the breakage of the 70mm infra-red in one of the goal arms (i.e. choice). Collect latency was defined as the time elapsed between the breakage of the 70mm infra-red beam in the chosen goal arm and the breakage of the 280mm infra-red in the chosen goal arm.

Errors committed during the post-reversal phase of testing were separated into below- and above- chance errors (Jones & Mishkin, 1972; Dias *et al.*, 1996; Chudasama & Robbins, 2003). Below-chance errors, reflecting perseveration, were defined as the number of errors committed below 50% correct response level (i.e. 5 correct responses out of 10 possible responses, at chance). If a subject made 3 correct choices, then the below-chance error would be 2 (=5 – 3); if a subject made more than 5 correct choices, then there were no below-chance errors. Above-chance errors, reflecting the formation of a new stimulus-reinforcer association, were defined as

the number of errors committed above 50% correct response level (i.e. 5 correct responses out of 10 possible responses, at chance). If a subject made 7 correct choices, then the above-chance error would be 3 (=10 – 7); if a subject made fewer than 5 correct choices, then the above-chance error would be 5.

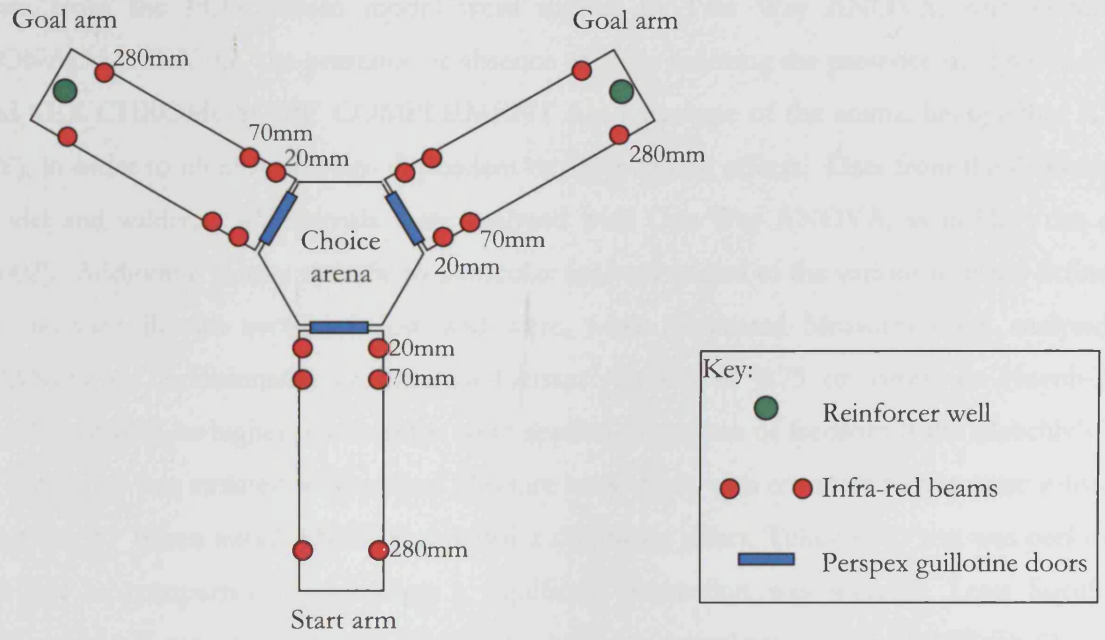


Figure 6.2.5a Schematic diagram of the simple visual discrimination and reversal learning apparatus. One goal arm was black and the other was white. In the acquisition and reversal learning phases, one of the reinforcer well contained the reinforcer which could not be seen from the choice arena.

6.2.6 Exclusion criteria and statistical analysis

Animals which did not sample the reinforcer during the 6-day reinforcer preference test were excluded from the preference test data analyses. Subjects were excluded from all analyses if the acquisition and re-acquisition performance criteria were not reached by the 40th and 60th sessions respectively. Animals which persistently did not drink the reinforcer during testing or develop side bias responding (defined as >85% responding to one side over eight consecutive days) were also excluded.

Learning in the acquisition phase was indexed by total number of errors committed up to reaching performance criterion. Perseverative responding and subsequent learning of the new contingency was indexed by below-chance and above-chance errors (to performance criterion), respectively. Latencies were averaged over the 10 trials to give mean latencies per session for each animal, and latencies at criterion were given by latencies averaged over the three criterion

sessions, for both acquisition and reversal phases. Data points which were deemed extreme outliers (on a box plot, data points that lay three times the interquartile range from the hinges were extreme outliers), as calculated by SPSS, were excluded.

Statistical analyses were performed using SPSS software (version 17, SPSS Inc., IBM, U.S.A.). Data from the FCG mouse model were subject to Two Way ANOVA, with factors of GONADAL SEX (i.e. the presence or absence of testis indexing the presence or absence of *Sry*) and SEX CHROMOSOME COMPLEMENT (i.e. karyotype of the animal being either XX or XY), in order to identify any *Sry*- dependent or independent effects. Data from the FCG mouse model and wildtype XY animals were analysed with One Way ANOVA, as in De Vries *et al.*, (2002). Additional factors specific to particular analyses related to the various tests are defined in the relevant Results section below and were, where Repeated Measures used, analysed by SPANOVA. Additionally, Greenhouse-Geisser (epsilon of 0.75 or lower) or Huynh-Feldt (epsilon of 0.75 or higher) corrections were applied to degrees of freedom if the Mauchly's Test of Sphericity was violated in Repeated Measure tests. Data with covariate factors were subject to ANCOVA. When initial ANOVA revealed a significant effect, Tukey HSD test was performed for *post hoc* comparisons, and when a significant interaction was revealed, Least Significant Difference adjustment was used for *post hoc* pairwise comparisons. Kruskal-Wallis One Way ANOVA was used for data not normally distributed. For all comparisons, p values of <0.05 were regarded as significant.

6.3 Results

6.3.1 Reinforcer preference test

Over the six-day test, the large majority of animals developed an increasing preference for 10% milk reinforcer over water. Data were subject to SPANOVA with Between Subject factors GONADAL SEX and SEX CHROMOSOME COMPLEMENT, and DAY (i.e. day three to six) as the Repeated Measures factor. Preference for milk increased significantly over days (effect of DAY, $F_{3,138}$ = 82.614, p<0.0001; **Figure 6.3.1a**), however, importantly, this preference did not vary between genotype groups (effect of GONADAL SEX, $F_{1,46}$ = 0.993, n.s.; SEX CHROMOSOME COMPLEMENT, $F_{1,46}$ = 0.308, n.s.; GONADAL SEX * SEX CHROMOSOME COMPLEMENT, $F_{1,46}$ = 1.000, n.s.).

Figure 6.3.1a

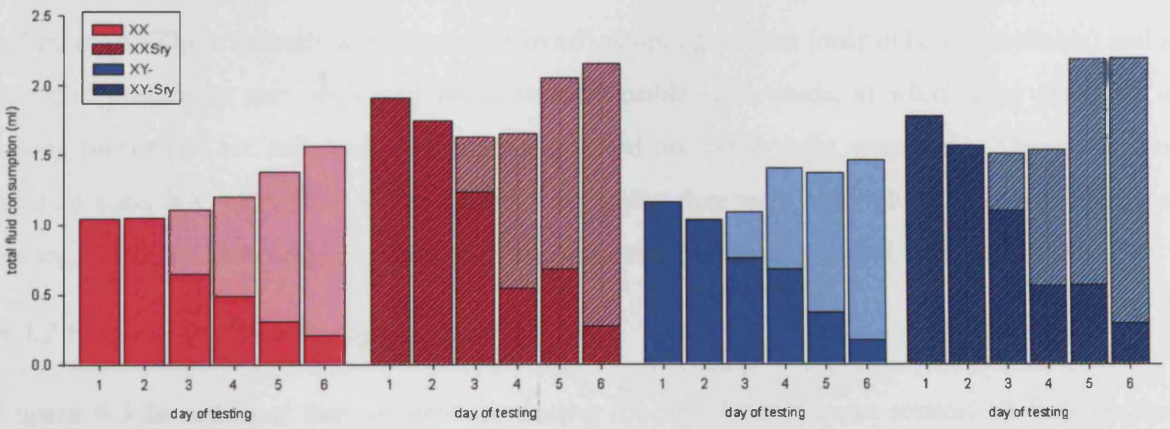


Figure 6.3.1a Total fluid consumption, separated into 10% milk reinforcer and water intake, over 6 days of testing. Preference for milk (light bars) over water (dark bars) increased over this period. Note that on days 1 and 2, only water was available. Data were presented as mean values.

Total fluid consumption data (**Figure 6.3.1b**) were subject to ANCOVA, with the above Between- and Within Subjects factors, and covariate of WEIGHT, as weight was likely to influence total fluid consumption. The total fluid consumed did not change significantly over the 6 days of testing (effect of DAY, $F_{4.681,210.652}$ = 1.948, n.s.); however, gonadal males were found to drink significantly more than gonadal females (effect of GONADAL SEX, $F_{1,45}$ = 9.085, p<0.01), irrespective of sex chromosome complement (effect of SEX CHROMOSOME COMPLEMENT, $F_{1,45}$ = 0.008, n.s.). Weight did not significantly affect the total fluid consumed by the animals (effect of WEIGHT, $F_{1,45}$ = 0.104, n.s.).

Figure 6.3.1b

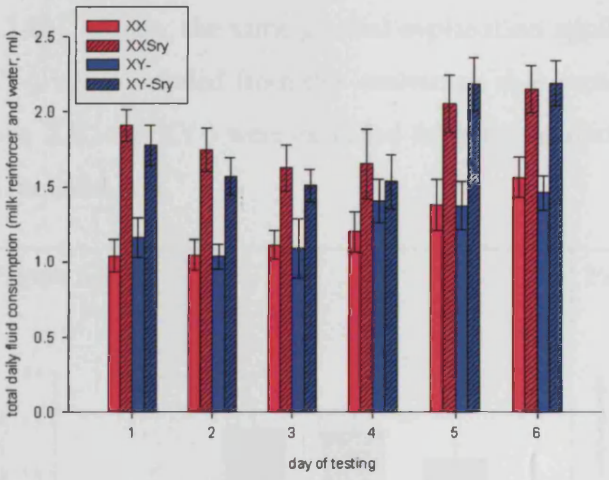


Figure 6.3.1b Total fluid consumption (including both milk and water) over six days of testing. Data are presented as mean values ± SEM.

A total of four animals were excluded from the reinforcer preference analysis. Two animals (one XY-, one XY-*Sry*) were not included as they did not drink the reinforcer during the six day habituation. These animals were given a 'forced' sampling session (only milk was available) and a 'normal' preference test (milk and water were available) afterwards, in which they displayed a strong preference for milk and therefore progressed on to the main maze task. The other two animals (one XX, one XY-) were excluded as, whilst they went through reinforcer preference testing, they failed to complete the maze task and were therefore excluded from all analyses.

6.3.2 Habituation to maze apparatus

Figure 6.3.2a indicates that, in general, during the initial habituation session all four groups explored the black and white goal arms approximately equally, with ratios of %time in black vs. white close to one. The statistical analysis did show subtle, complex effects on choice arm preference, whereby there was a significant interaction of GONADAL SEX × SEX CHROMOSOME COMPLEMENT ($F_{1,44}$ = 4.538, $p<0.05$), but no significant main effect of GONADAL SEX ($F_{1,44}$ = 0.546, n.s.) nor SEX CHROMOSOME COMPLEMENT ($F_{1,44}$ = 0.302, n.s.). Broadly, this means that in gonadally identical animals, sex chromosome complement can have a modulating influence, in that there was a preference for the black arm shown by the animals where the *Sry* transgene was expressed in the XX karyotype ($p<0.05$) and when the *Sry* transgene was expressed in the XY karyotype, there tended to be an opposite direction of effect (without actually spending more time in the white arm; however, *post hoc* pairwise comparison was not significant). Similarly, a significant interaction of GONADAL SEX × SEX CHROMOSOME COMPLEMENT was found in the ratio of black arm entries to

white arm entries ($F_{1,44}$ = 5.202, $p<0.05$), with no significant main effect of GONADAL SEX ($F_{1,44}$ = 0.712, n.s.) nor SEX CHROMOSOME COMPLEMENT ($F_{1,44}$ = 0.585, n.s., **Figure 6.3.2b**). Again, the same general explanation applies. A total of three subjects (two XXs, one XY-) were excluded from this analysis as they were extreme outliers. Additionally, two animals (one XX, one XY-) were excluded from the analysis as they subsequently failed to complete the main maze task.

Figure 6.3.2a

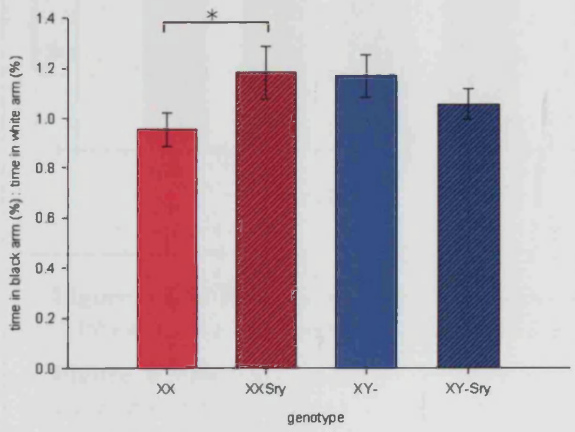


Figure 6.3.2b

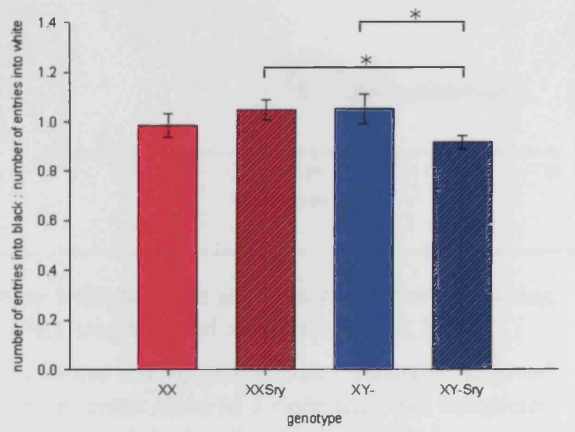


Figure 6.3.2a Ratio of percentage time spent in black arm to percentage time spent in white arm. The percentages took in account time spent in the middle choice area and in the start arm. Data are presented as mean values ± SEM. * $p<0.05$

Figure 6.3.2b Ratio of number of black arm entries to number of white arm entries. Data are presented as mean values ± SEM. * $p<0.05$

6.3.3 Acquisition of the stimulus-reinforcer contingency (errors)

The total number of errors made in reaching acquisition performance criterion was subject to Three Way ANOVA, with Between Subject factors GONADAL SEX, SEX CHROMOSOME COMPLEMENT and ARM (i.e. whether the reinforcer arm colour for a subject was black or white). The factor ARM was included as there was a possibility of differential learning between black and white reinforcer arms. **Figure 6.3.3a** shows that gonadal male subjects acquired the initial stimulus-reinforcer contingency with significantly fewer errors than gonadal females (effect of GONADAL SEX, $F_{1,44}$ = 5.680, $p<0.05$), irrespective of SEX CHROMOSOME COMPLEMENT ($F_{1,44}$ = 0.001, n.s.,). The reinforcer arm colour did not influence the number

of acquisition errors committed (effect of ARM, $F_{1,44} = 0.001$, n.s.). Additionally, there were no significant interactions between the 3 factors[19].

Figure 6.3.3a

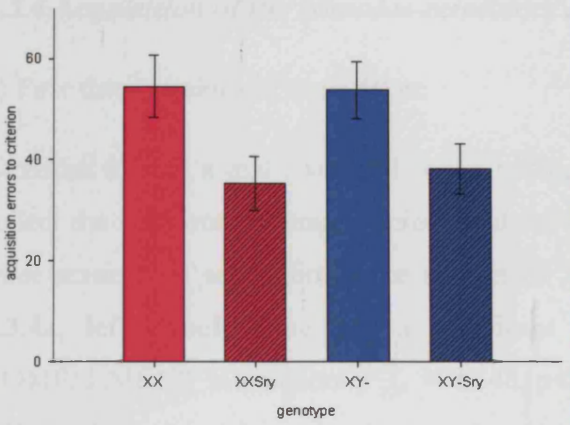


Figure 6.3.3b

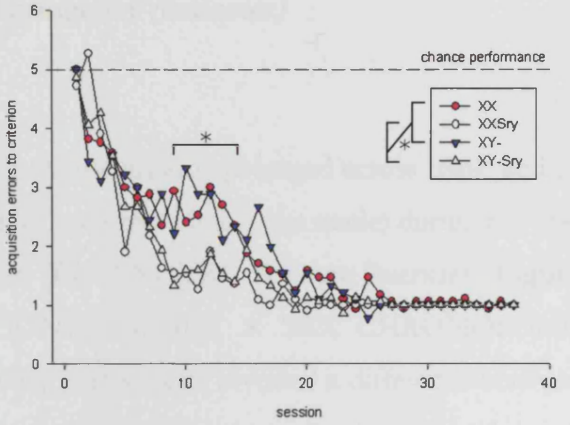


Figure 6.3.3a Total number of errors committed in order to reach acquisition criterion (achieving ≥85% correct every day for 3 consecutive days). Data are presented as mean values ± SEM.

Figure 6.3.3b Total number of errors across sessions during acquisition. Animals achieved acquisition criterion at various rates and were given an error score of 1 once they had completed acquisition, in order to keep the number of subjects constant across sessions. Data are presented as mean values. * $p<0.05$

Data on acquisition errors made on individual sessions are shown in **Figure 6.3.3b**. These more detailed data were subject to SPANOVA with Between Subject factors of GONADAL SEX and SEX CHROMOSOME COMPLEMENT, and Repeated Measures factor SESSION (Session 1-37). Importantly, all four groups started at an equivalent level of performance at the beginning of training (approximately chance – 50%) and reflecting learning of the task all four groups made significantly fewer errors as training progressed (effect of SESSION, $F_{11.154,535.407} = 48.493$, $p<0.001$); consistent with the overall mean error data above, gonadal males were found to make fewer errors than gonadal females (effect of GONADAL SEX, $F_{1,48} = 6.739$, $p<0.05$), irrespective of SEX CHROMOSOME COMPLEMENT ($F_{1,48} = 0.023$, n.s.). There was no interaction between the Between Subject factors (GONADAL SEX × SEX CHROMOSOME COMPLEMENT, $F_{1,48} = 0.082$, n.s.). The rate of learning between gonadal males and gonadal females differed significantly across the training sessions (SESSION × GONADAL SEX

[19] F values for interactions are as follows: SEX CHROMOSOME COMPLEMENT × GONADAL SEX, $F_{1,44} = 0.032$; SEX CHROMOSOME COMPLEMENT × ARM, $F_{1,44} = 2.571$; GONADAL SEX × ARM, $F_{1,44} = 0.064$; SEX CHROMOSOME COMPLEMENT × GONADAL SEX × ARM, $F_{1,44} = 0.200$; all n.s..

interaction, $F_{11.154,535.407} = 3.055$, $p<0.001$); the number of errors committed at the beginning and end of the acquisition period were similar in all subjects, but differed significantly between gonadal males and females between sessions 9 and 14 ($p<0.05$ by *post hoc* analysis) with more errors being made by gonadal females than males.

6.3.4 Acquisition of the stimulus-reinforcer contingency (latencies)

(i) First three sessions in acquisition:

Latencies to start a trial (averaged across trials), to choose an arm (averaged across trials) and to collect the reinforcer (averaged across trials in which a correct choice was made) during the first three sessions of acquisition were subject to Two Way ANOVA. For start latencies (**Figure 6.3.4a, left panel**) there was a significant GONADAL SEX × SEX CHROMOSOME COMPLEMENT interaction ($F_{1,47} = 4.148$, $p<0.05$); *post hoc* tests revealed a difference between XX and XY- gonadal females, i.e. sex chromosome complement appeared to have a modulating effect on start latencies, in the absence of a *Sry* transgene ($p<0.05$). There were no significant main effects of GONADAL SEX ($F_{1,47} = 0.334$, n.s.) or effect of SEX CHROMOSOME COMPLEMENT ($F_{1,47} = 1.062$, n.s.).

Figure 6.3.4a

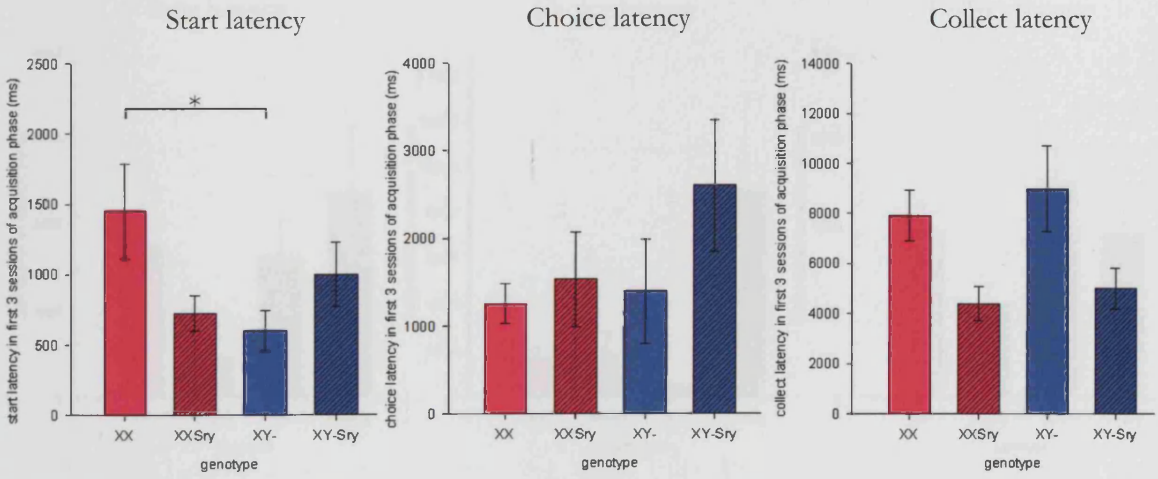


Figure 6.3.4a (Left to right) Start, choice and collect latencies (ms) at the start of acquisition phase. Data were averaged from the first 3 consecutive sessions in acquisition. Data are presented as mean values ± SEM. * $p<0.05$

For choice latencies over the first three sessions of the acquisition stage (**Figure 6.3.4a, centre panel**), there were no significant effects of GONADAL SEX ($F_{1,47} = 1.656$, n.s.) nor SEX CHROMOSOME COMPLEMENT ($F_{1,47} = 1.112$, n.s.) nor any interactions between the two factors ($F_{1,47} = 0.653$, n.s.).

For collect latencies over the first three sessions of acquisition stage (**Figure 6.3.4a, right panel**), gonadal male subjects collected the reinforcer significantly quicker than gonadal females (effect of GONADAL SEX, $F_{1,47}$ = 11.814, $p<0.005$), irrespective of SEX CHROMOSOME COMPLEMENT ($F_{1,47}$ = 0.588, n.s.). There was no interaction between the two factors (GONADAL SEX × SEX CHROMOSOME COMPLEMENT, $F_{1,47}$ = 0.041, n.s.).

(ii) Start, choice and collect latencies on session nine:

Session nine was the first session in which a significant pair-wise difference was observed between gonadal males and gonadal females in terms of errors (indexing learning; **Figure 6.3.3b**), therefore I decided to examine the latencies on this session which might give an additional measure of learning. All mice were still in the acquisition phase on session 9. As data were not normally distributed, the two Between Subject factors GONADAL SEX and SEX CHROMOSOME COMPLEMENT were collapsed to give a single factor of GENOTYPE, so that latencies to start (averaged across every trial), to choose an arm (averaged across every trial) and to collect the reinforcer (averaged across trials in which a correct choice was made) during session nine could be analysed using Kruskal-Wallis One Way ANOVA.

Figure 6.3.4b

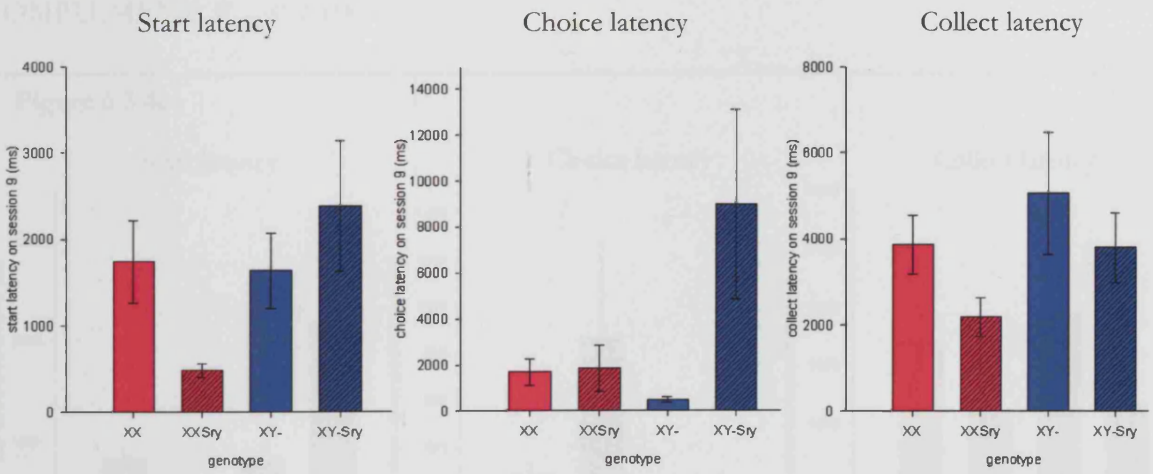


Figure 6.3.4b (Left to right) Start, choice and collect latencies (ms) on session nine. Data are presented as mean values ± SEM.

On session nine, subjects generally displayed longer start and choice latencies, and shorter collect latencies, compared to the first three sessions in acquisition phase (**Figure 6.3.4b**), which suggests that subjects were beginning to acquire the stimulus-reinforcer contingency, and therefore took longer to deliberate on their choices, and were quicker to collect the increasingly expected reinforcer after they had made a correct choice. For start, choice and collect latencies,

subjects did not vary between each other (start, $H_3 = 6.795$, n.s.; choice, $H_3 = 1.125$, n.s.; collect, $H_3 = 5.232$, n.s.).

(iii) Three sessions at stable acquisition performance criterion:

Latencies to start (averaged across every trial), to choose an arm (averaged across every trial) and to collect the reinforcer (averaged across trials in which a correct choice was made) during the three consecutive performance criterion sessions were subject to Two Way ANOVA. There was no effect of GONADAL SEX on start latencies at performance ($F_{1,44} = 1.562$, n.s.), nor of SEX CHROMOSOME COMPLEMENT ($F_{1,44} = 3.354$, n.s.) and nor was there any interaction between the two factors (GONADAL SEX × SEX CHROMOSOME COMPLEMENT, $F_{1,44} = 3.46$, n.s.; **Figure 6.3.4c, left panel**).

Consistent with animals learning the task to a high degree of stimulus control, choice latencies at acquisition performance criterion were dramatically shorter across all four groups compared to the beginning of training (74% average percentage decrease; **Figure 6.3.4c, centre panel**). There was no effect of GONADAL SEX on choice latencies in this phase of the task ($F_{1,39} = 2.485$, n.s.), nor of SEX CHROMOSOME COMPLEMENT ($F_{1,39} = 3.109$, n.s.). There was no interaction between the two factors (GONADAL SEX × SEX CHROMOSOME COMPLEMENT, $F_{1,39} = 2.106$, n.s.).

Figure 6.3.4c

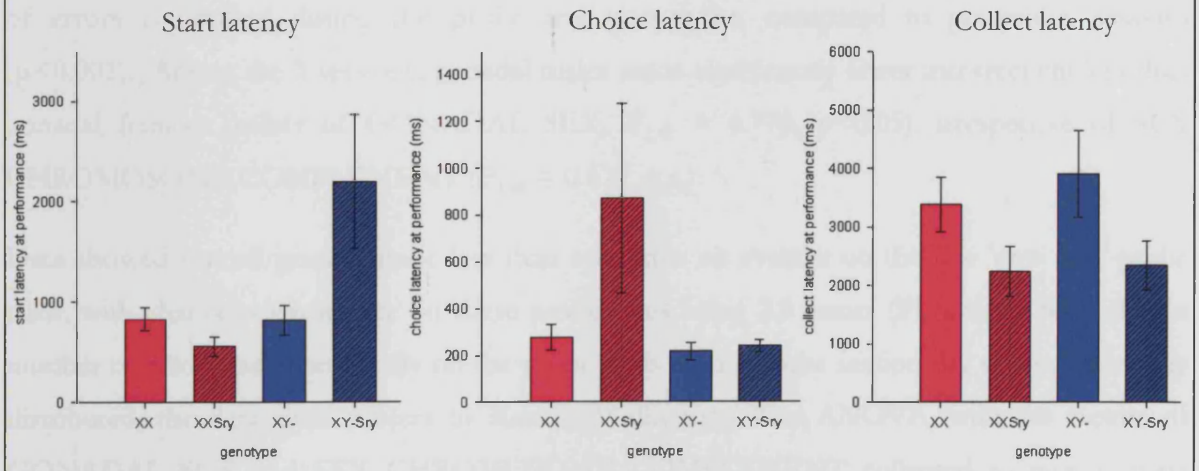


Figure 6.3.4.c (Left to right) Start, choice and collect latencies (ms) at performance. Data were averaged from the 3 consecutive sessions in which animals reached acquisition criterion. Data are presented as mean values ± SEM.

Collect latencies, like choice latencies, were markedly shorter at performance criterion than at the start of acquisition phase across all four groups (54% average percentage decrease; **Figure 6.3.4c,**

right panel). The pattern of data at start of acquisition was maintained at performance criterion such that gonadal male subjects collected the reinforcer significantly quicker than gonadal females (effect of GONADAL SEX, $F_{1,46} = 7.035$, $p<0.05$). There was no effect of SEX CHROMOSOME COMPLEMENT on this measure ($F_{1,46} = 0.38$, n.s.) and no interaction between the two factors (GONADAL SEX × SEX CHROMOSOME COMPLEMENT, $F_{1,46} = 0.163$, n.s.).

6.3.5 Odour usage

At stable performance, a probe session was performed to determine whether subjects were using olfactory cues to guide their behaviour. If subjects were using olfactory cues exclusively, one would expect their performance on trials where new arms were introduced to be at, or around, chance level (i.e. 2.5 correct choices, out of five probe trials). Number of errors made (maximum of ten, chance performance at five errors) from three consecutive days (pre-probe, probe and post-probe) gave an index as to how much disruption the use of 'new' arms had on performance. As these data were not normally distributed, data were transformed with $\sqrt{x+0.5}$ for analysis which helped to reduce the skewness. Data from three consecutive days (**Figure 6.3.5a**) was subject to SPANOVA, with Between Subject factors of GONADAL SEX and SEX CHROMOSOME COMPLEMENT, and Repeated Measures factor of DAY (pre-probe, probe and post-probe days). There was a significant difference in total errors between sessions (effect of DAY, $F_{2,96} = 20.245$, $p<0.001$); *post hoc* revealed an increased number of errors committed during the probe and post-probe, compared to pre-probe, sessions ($p<0.001$). Across the 3 sessions, gonadal males made significantly fewer incorrect choices than gonadal females (effect of GONADAL SEX, $F_{1,48} = 6.775$, $p<0.05$), irrespective of SEX CHROMOSOME COMPLEMENT ($F_{1,48} = 0.672$, n.s.).

Data showed that all groups made less than one error on average on the five 'new arm' probe trials, with chance performance on these probe trials being 2.5 errors (**Figure 6.3.5b**). As the number of error made specifically on the probe trials on the probe session day was not normally distributed, the data were subject to Kruskal-Wallis One Way ANOVA, with the factors of GONADAL SEX and SEX CHROMOSOME COMPLEMENT collapsed to give a single Between Subject factor of GENOTYPE. There was no difference in the number of errors made between genotype groups ($H_3 = 1.729$, n.s.).

There was a general small increase in errors on (probe) trials 2, 4, 6, 8 and 10 on the probe day and on post-probe day (average errors on probe day: 0.75; on post-probe day: 0.7) compared to

performance on the same trials on the pre-probe day (average error on pre-probe day: 0.3). All data considered, I would conclude that there was no usage of odour cues to guide choice behaviour, as number of errors made on probe trials (with new arms) was substantially lower than chance performance (2.5 errors). The small effects on performance were evident on probe day and were carried over to post-probe day. Note that on post-probe day, the usual goal arms were used, but effects on performance persisted; if animals were indeed using odour cues to guide behaviour, one would expect no effects on performance as animals returned to using the odour cues available, and thus, I argue that the effects on performance on probe day were more likely to be caused by general task disruption rather than the removal of behaviour-guiding odour cues.

Figure 6.3.5a

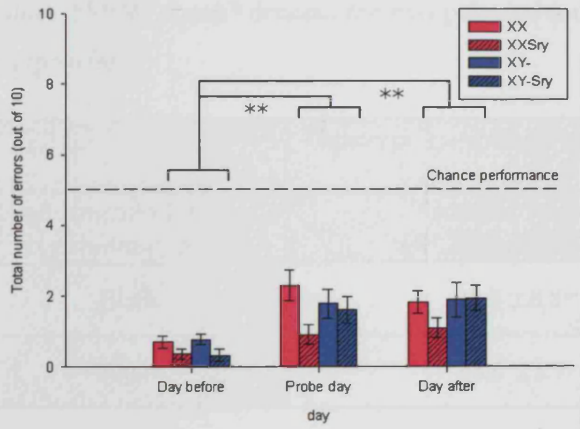


Figure 6.3.5b

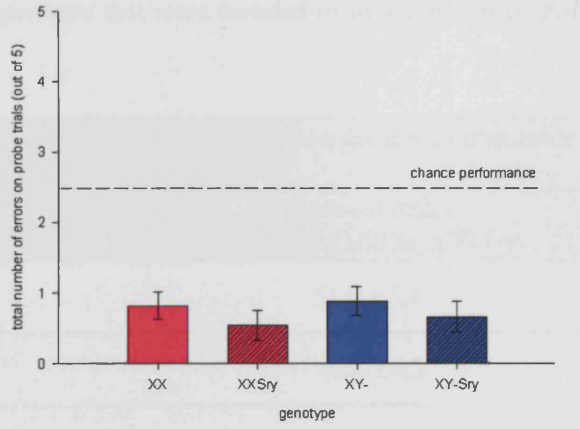


Figure 6.3.5a Total number of errors out of a maximum ten across three consecutive sessions; pre-probe, probe and post-probe. Total of five errors would suggest subjects were performing at chance. Data are presented as mean values ± SEM. ** $p<0.001$

Figure 6.3.5b Total number of errors made on probe trials out of a maximum 5 within the probe session. Total of 2.5 errors would suggest subjects were performing at chance. Data are presented as mean values ± SEM.

6.3.6 Reversal of the stimulus-reinforcer contingency (errors)

(i) Total errors:

As a general index of reversal learning, total errors in obtaining the re-acquisition performance criterion (**Figure 6.3.6a**, **left panel**) were analysed with Three Way ANOVA, with Between Subject factors of GONADAL SEX, SEX CHROMOSOME COMPLEMENT and ARM (i.e. whether the reinforcer arm colour for a subject was black or white). The analysis showed gonadal males made fewer errors than gonadal females (effect of GONADAL SEX, $F_{1,44} = 6.481$,

$p<0.05$) irrespective of SEX CHROMOSOME COMPLEMENT ($F_{1,44} = 0.241$, n.s.); there was no significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,44} = 3.691$, n.s.). However, animals initially trained with the black arm as the reinforcer arm in the acquisition phase (reversed to white arm) made significantly more errors than those trained with the white arm (reversed into black arm) (effect of ARM, $F_{1,44} = 12.437$, $p<0.01$). Additionally, there was a significant interaction between GONADAL SEX and ARM ($F_{1,44} = 10.359$, $p<0.01$) and *post hoc* tests suggest that while arm colour affected performance of gonadal female subjects, with more errors committed if the gonadal females were initially trained with a black reinforcer arm; arm colour did not affect gonadal male subject performance (**Table 6.3.6i**).

Table 6.3.6i Table detailing the number of errors made in obtaining the re-acquisition performance criterion in reversal phase, separated into genotype groups and arm colour. Data are presented as mean values ±SEM. [a] and [b] denotes the two pairwise comparisons that were revealed to be significant by *post hoc* ($p<0.001$).

	Genotype groups and total errors in obtaining the re-acquisition performance criterion in reversal phase	
Goal arm colour trained in acquisition phase	Gonadal females (40,XX and 40,XY-)	Gonadal males (40,XX*Sry* and 40,XY-*Sry*)
Black	81.2 ±8.8[ab]	51.6 ±3.4[a]
White	43.6 ±3.7[b]	46.6 ±4.3

Figure 6.3.6a

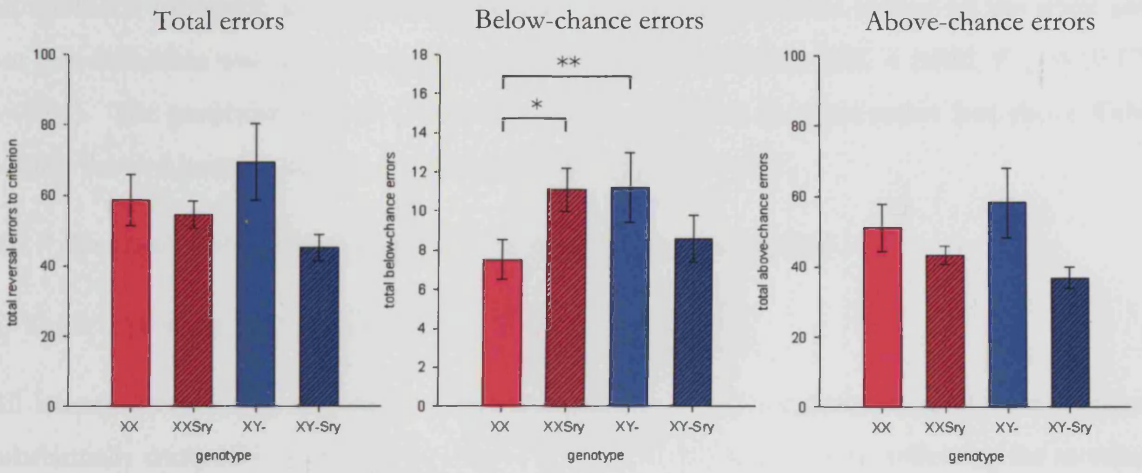


Figure 6.3.6a (Left) Total errors to re-acquisition performance criterion (achieving ≥85% correct every day for 3 consecutive days). The number of errors could be separated into total below-chance (centre) and above-chance (right) errors. Data are presented as mean values ± SEM. * p=0.05; ** p<0.05

(ii) Below-chance (perseverative) errors:

In order to gain more information about which specific strategies might be giving rise to the effects found in total errors analysis above, total errors were sub-divided into below- (<50%) and above-chance (>50%) errors reflecting perseveration (and the attendant requirement to inhibit a pre-potent response) and acquisition of a novel stimulus-reinforcer association, respectively. With regard to below-chance reversal errors (**Figure 6.3.6a**, **centre panel**), animals performed equivalently (effect of GONADAL SEX, $F_{1,44}$ = 0.051, n.s.; effect of SEX CHROMOSOME COMPLEMENT, $F_{1,44}$ = 0.337, n.s.; effect of ARM, $F_{1,44}$ = 2.259, n.s.). However, there was a significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,44}$ = 6.450; p<0.05) and *post hoc* tests revealed that XX*Sry* and XY- animals made significantly more below-chance errors than XX animals (p=0.05 and p<0.05 respectively).

(iii) Above-chance errors:

Following reversal and re-acquisition of the new stimulus-reinforcer contingency (**Figure 6.3.6a**, **right panel**), gonadal males committed significantly fewer errors than gonadal females (effect of GONADAL SEX, $F_{1,44}$ = 8.199, p<0.01), irrespective of SEX CHROMOSOME COMPLEMENT ($F_{1,44}$ =0.182, n.s.). There was no interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,44}$ = 2.224, n.s.). Animals previously trained on the black arm during acquisition phase committed significantly more errors than those trained on the

white arm (effect of ARM, $F_{1,44}$ = 12.462, $p<0.01$); additionally, gonadal females initially trained on the black arm made significantly more errors than gonadal females trained on the white arm, but this difference was not present in gonadal males (GONADAL SEX × ARM, $F_{1,44}$ = 10.178, $p<0.01$). The particular pattern of results is similar to that in the total errors (see above **Table 6.3.6i**); the two pairwise comparisons were significant to $p<0.001$.

6.3.7 Reversal of the stimulus-reinforcer contingency (latencies)

(i) Three days immediately subsequent to contingency reversal:

All latency measures during the three days subsequent to contingency reversal were increased substantially compared to those at performance of the acquisition phase, reflecting the increased difficulty of the task, in particular with respect to having to inhibit the well learnt (i.e. pre-potent) previously correct response in favour of responding to the now correct but previously incorrect choice. Latencies to start a trial (averaged across trials), to choose an arm (averaged across trials) and to collect the reinforcer (averaged across trials in which a correct choice was made) during the three sessions were subject to Two Way ANOVA. With regard to start latencies (**Figure 6.3.7a, left panel**), gonadal males were quicker to leave the start arm than females (effect of GONADAL SEX, $F_{1,45}$ = 7.749, $p<0.01$); additionally, animals with a XX karyotype displayed shorter start latencies than animals with a XY karyotype (effect of SEX CHROMOSOME COMPLEMENT, $F_{1,45}$ = 5.605, $p<0.05$). There was no significant interaction between the two factors ($F_{1,45}$ = 0.784, n.s.).

For choice latencies (**Figure 6.3.7a, centre panel**), there were no group effects (GONADAL SEX, $F_{1,42}$ = 0.058, n.s.; SEX CHROMOSOME COMPLEMENT, $F_{1,42}$ = 0.048, n.s.) nor interaction between the two factors ($F_{1,42}$ =0.004, n.s.).

For collect latencies (**Figure 6.3.7a, right panel**), animals with a XX karyotype were quicker to collect the reward than XY karyotype animals (effect of SEX CHROMOSOME COMPLEMENT, $F_{1,46}$ = 4.673, $p<0.05$), irrespective of gonadal status (effect of GONADAL SEX, $F_{1,46}$ = 1.863, n.s.). There was a significant interaction between GONADAL SEX and SEX CHROMOSOME COMPLEMENT ($F_{1,46}$ = 7.674, $p<0.01$) and *post hoc* tests revealed a difference between XY- and XX, and between XY- and XY-*Sry* ($p<0.01$).

Figure 6.3.7a

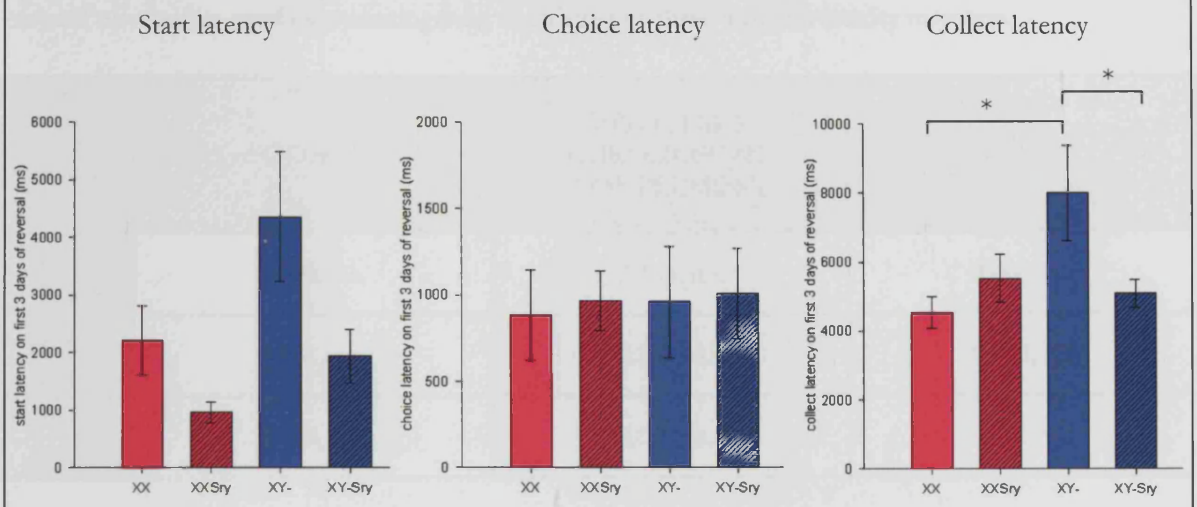


Figure 6.3.7a (left to right) Start, choice and collect latencies on the three days subsequent to reversal. Data are presented as mean values ± SEM. * $p<0.01$

(ii) Three sessions at stable re-acquisition performance criterion:

During the three consecutive sessions in which animals reached re-acquisition criterion in the reversal phase, animals regained high performance competency after the reversal of stimulus-reinforcer contingencies (**Figure 6.3.7b**) which was reflected by the shorter start and choice latencies compared to those at the beginning of the reversal phase (**Figure 6.3.7.a**). Animals did not differ significantly on any of the three latency measures (**Table 6.3.7i**).

Figure 6.3.7b

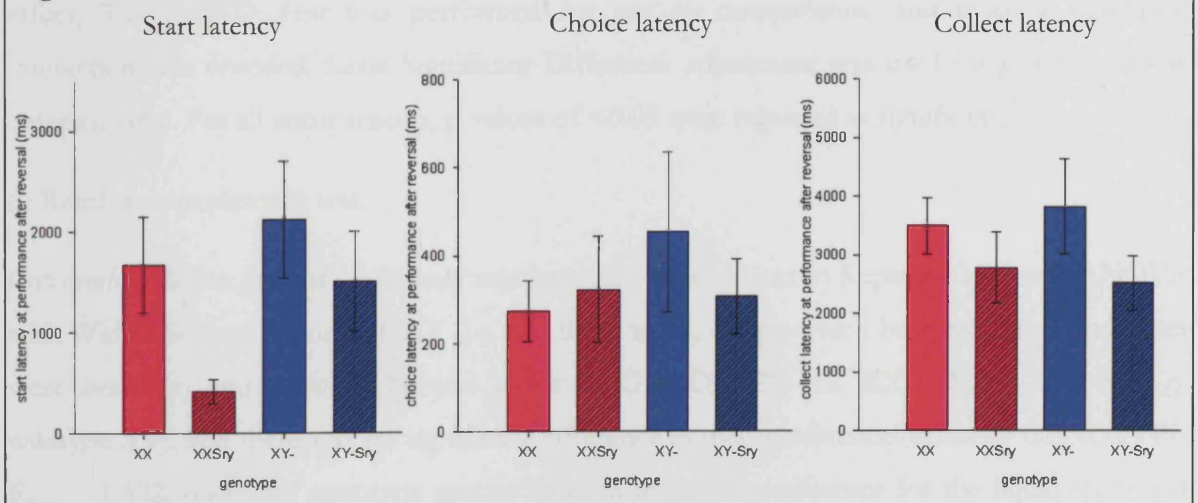


Figure 6.3.7b (left to right) Start, choice and collect latencies at performance after reversal. Data were averaged from the three consecutive sessions in which animals reached reversal criterion. Data are presented as mean values ± SEM.

Table 6.3.7i Statistical analysis of performance across three consecutive days at performance after reversal of stimulus-reinforcer contingency, as indexed by three different latency measures.

Latency measures	Effect of GONADAL SEX	Effect of SEX CHROMOSOME COMPLEMENT	Interaction of GONADAL SEX × SEX CHROMOSOME COMPLEMENT
Start $F_{1,48}$	3.500, n.s.	2.364, n.s.	0.408, n.s.
Choice $F_{1,43}$	0.211, n.s.	0.588, n.s.	0.833, n.s.
Collect $F_{1,47}$	3.040, n.s.	0.003, n.s.	0.261, n.s.

6.3.8 Additional data from XY wildtype mice

Wildtype XY male MF1 mice were also subject to the simple visual discrimination and reversal learning task. I wanted to compare the performance of this genotype group to that of mice generated in the FCG cross, to examine how closely the behavioural performance of mice with a *Sry* transgene from the FCG cross (of unknown copy number, and of unknown insertion site) resembled that of mice with an endogenous *Sry* gene (i.e. wildtype XY). Data in this section were subject to One Way ANOVA, with the Between Subject factor of GENOTYPE (i.e. XX, XX*Sry*, XY-, XY-*Sry* and wildtype XY subjects). Additional factors particular to the analysis, if any, are detailed in the relevant sections below. When initial ANOVA revealed a significant effect, Tukey HSD Test was performed for *post hoc* comparisons, and when a significant interaction was revealed, Least Significant Difference adjustment was used for *post hoc* pairwise comparisons. For all comparisons, p values of <0.05 were regarded as significant.

(i) Reinforcer preference test:

Comparing all five groups of animals together, data were subject to Repeated Measures ANOVA with Within Subject factor of DAY (i.e. day three to six, during which both reinforcer and water were available) and Between Subject factor of GENOTYPE (i.e. XX, XX*Sry*, XY-, XY-*Sry*, wildtype XY), and there was no significant difference in their preference (effect of GENOTYPE, $F_{4,56} = 1.322$, n.s.). All genotype groups showed increased preference for the liquid reinforcer over water during the testing period (effect of DAY, $F_{3,168} = 100.861$, $p<0.001$; **Figure 6.3.8a**, **Figure 6.3.1a**) and there was no significant interaction between DAY and GENOTYPE ($F_{12,168} = 1.561$, n.s.)

Figure 6.3.8a

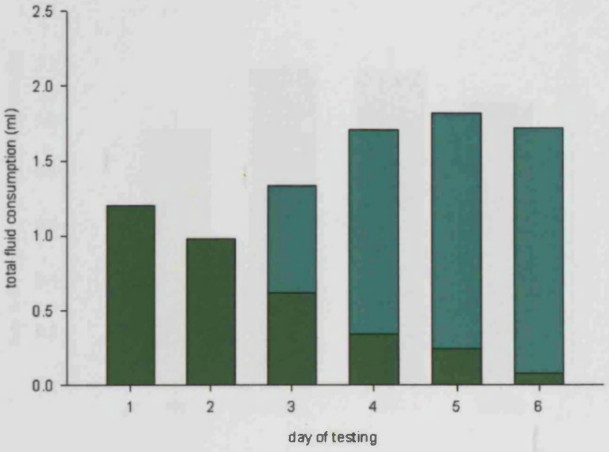


Figure 6.3.8a Total fluid consumption, separated into 10% milk reinforcer and water intake, over 6 days of testing, for wildtype XY male mice. Preference for milk (light bars) over water (dark bars) increased over this period. Note that on days 1 and 2, only water was available. Data were presented as mean values.

For data on FCG animals, please refer to **Figure 6.3.1a**.

(ii) Habituation to maze apparatus:

Figure 6.3.8b and **Figure 6.3.8c** show the habituation data from wildtype XY males next to that from the FCG animals; the duration spent in and number of entries into black and white arms were calculated as ratios. There were no significant differences between the genotype groups in both duration ratio (effect of GENOTYPE, $F_{4,57} = 1.119$, n.s.) and entries ratio (effect of GENOTYPE, $F_{4,57} = 1.556$, n.s.).

Figure 6.3.8b

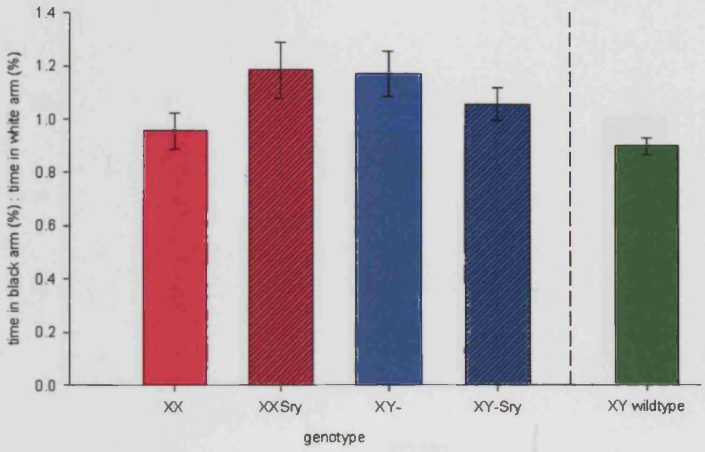


Figure 6.3.8b Ratio of percentage time spent in black arm to percentage time spent in white arm. The percentages took in account time spent in the middle choice area and in the start arm. Data are presented as mean values ± SEM.

Figure 6.3.8c

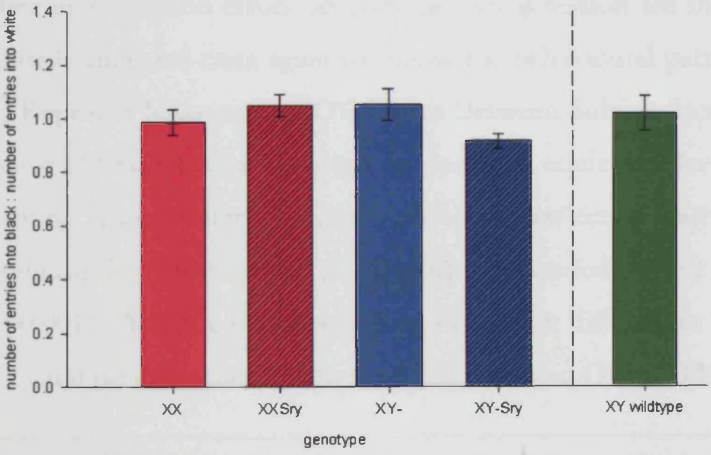


Figure 6.3.8c Ratio of number of black arm entries to number of white arm entries. Data are presented as mean values ± SEM.

(iii) Total number of errors made in reaching acquisition performance criterion:

With regard to the total number of errors made in reaching acquisition performance criterion, gonadal males (XX*Sry* and XY-*Sry*) committed significantly fewer errors, notably between sessions 9-14, than gonadal females (XX and XY-) in the FCG mouse model comparison (see section **6.3.3** above). As suggested in **Figure 6.3.8d**, wildtype XY animals appeared to perform comparably to the gonadal females from the FCG mouse model, giving rise to a main effect of GENOTYPE ($F_{4,58}$ = 2.534, p=0.05). *Post hoc* test did not reveal any significant pairwise comparisons.

Figure 6.3.8d

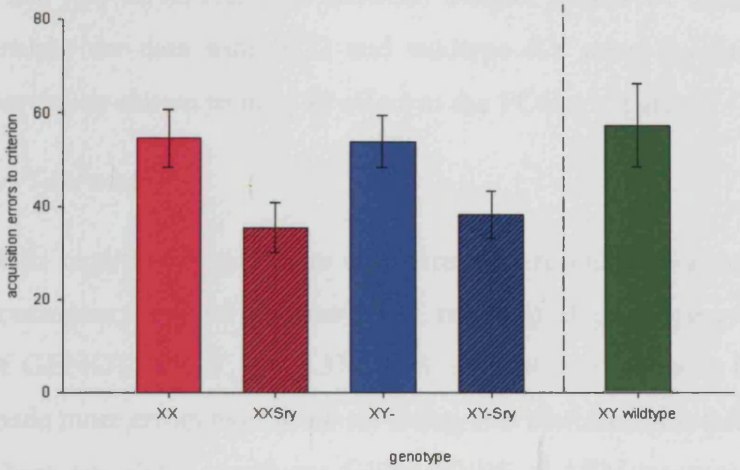


Figure 6.3.8d Total number of errors made in reaching acquisition performance criterion for the FCG mice and wildtype XY animals. Data are presented as mean values ± SEM.

Data in acquisition errors on each individual session are shown in **Figure 6.3.8e**. Wildtype XY animals appeared once again to follow the behavioural pattern of gonadal females. As indicated by Repeated Measures ANOVA with Between Subject factor GENOTYPE and Within Subject factor SESSION, all five groups started at an equivalent level of performance at the beginning of training (approximately chance ~50%) and reflecting learning of the task all five groups made significantly fewer errors as training progressed (effect of SESSION, $F_{11.303,655.554}$= 59.451, p<0.001). Analysis also revealed no significant differences between GENOTYPE ($F_{4,58}$ = 2.284, n.s.) and no interactions between SESSION and GENOTYPE ($F_{45.211,655.554}$ = 1.241, n.s.).

Figure 6.3.8e

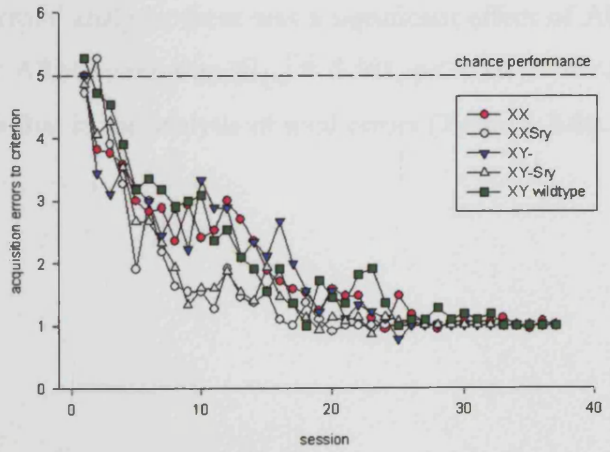


Figure 6.3.8e Total number of errors across sessions during acquisition committed by the FCG animals and wildtype XY males. Animals achieved acquisition criterion at various rates and were given an error score of 1 once they had completed acquisition, in order to keep the number of subjects constant across sessions. Data are presented as mean values.

(iv) Errors committed in reaching re-acquisition performance criterion after contingency reversal:

Two Way ANOVA, with Between Subject factors of GENOTYPE and ARM, was used to analyse the data with FCG and wildtype XY mice; the factor ARM was included as it was previously shown to have an effect in the FCG comparison.

(a) Total errors:

With regard to total errors committed in reaching re-acquisition performance criterion after contingency reversal (**Figure 6.3.8f, top left**), all genotype groups performed equivalently (effect of GENOTYPE, $F_{4,53} = 2.370$, n.s.), and subjects reversing into the white arm (from the black) made more errors than those reversing into the black arm (effect of ARM, $F_{1,53} = 5.096$, $p<0.05$). There was also a significant GENOTYPE × ARM interaction ($F_{4,53} = 5.246$, $p<0.001$); *post hoc* comparisons can be found in **Table 6.3.8i**.

(b) Below-chance (perseverative) errors:

Subjects made equivalent numbers of below-chance errors (**Figure 6.3.8f, top right**; effect of GENOTYPE, $F_{4,53} = 2.008$, n.s.). Arm colour did not affect the number of errors made (effect of ARM, $F_{1,53} = 0.905$, n.s.) and there was no significant GENOTYPE × ARM interaction ($F_{4,53} = 1.174$, n.s.).

(c) Above-chance (formation of new stimulus-reinforcer contingencies) errors:

Genotype groups performed equivalently (effect of GENOTYPE, $F_{4,53} = 2.506$, n.s.) in terms of above-chance errors (**Figure 6.3.8f, bottom left**). Similar to the pattern of results in the 'total errors' analysis, there was a significant effect of ARM ($F_{1,53} = 5.201$, $p<0.05$) and GENOTYPE × ARM interaction ($F_{4,53} = 5.381$, $p<0.001$). *Post hoc* analysis yielded the same pattern of results as that in the analysis of total errors (**Table 6.3.8i**).

Figure 6.3.8f

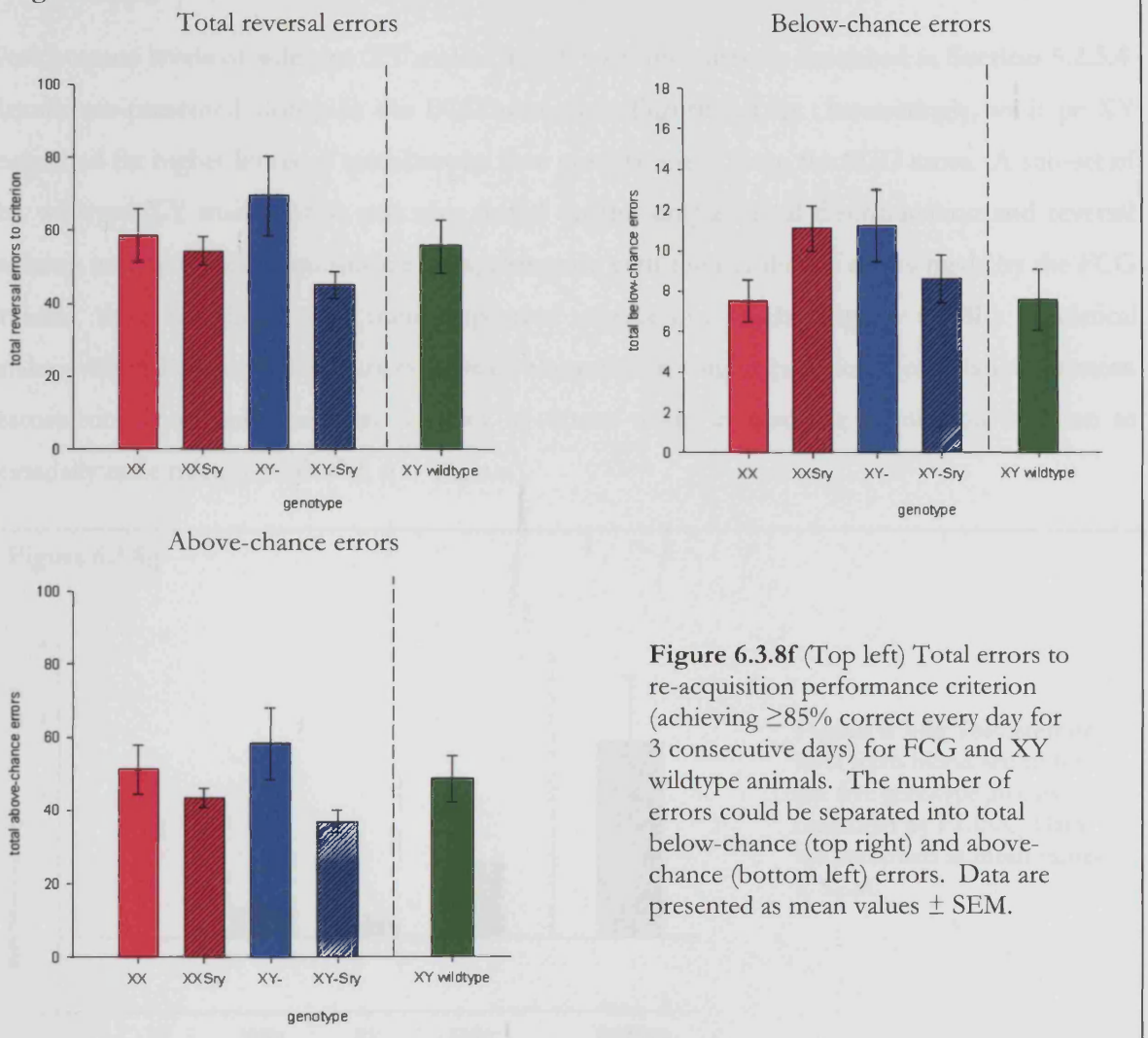


Figure 6.3.8f (Top left) Total errors to re-acquisition performance criterion (achieving ≥85% correct every day for 3 consecutive days) for FCG and XY wildtype animals. The number of errors could be separated into total below-chance (top right) and above-chance (bottom left) errors. Data are presented as mean values ± SEM.

Table 6.3.8i *Post hoc* analysis of GENOTYPE × ARM interaction in total and above-chance errors committed in reaching re-acquisition performance criterion. Only significant differences ($p<0.05$) are reported here.

Colour of arm initially reinforced in acquisition phase → colour of arm reinforced after contingency reversal	Significant *post hoc* differences
Black → white	Gonadal females (XX & XY-) differed significantly from gonadal males (XX*Sry*, XY-*Sry* and XY wildtype).
White →black	XY wildtype animals differed significantly from XX and XY-*Sry*.
Genotype group	
XX and XY-	For both of these genotype groups, total errors made in 'black → white' were higher than that in 'white → black'.

(v) Blood plasma testosterone level and its correlation with acquisition errors

Testosterone levels of wildtype XY males (n=10) were measured as described in **Section 5.2.3.4**. Results are presented alongside the FCG animals in **Figure 6.3.8g**. Interestingly, wildtype XY males had far higher levels of testosterone than gonadal males from the FCG cross. A sub-set of the wildtype XY males (n=4) was also tested on the simple visual discrimination and reversal learning task, and their acquisition errors, alongside with the number of errors made by the FCG animals, were correlated with their respective testosterone levels (**Figure 6.3.8h**) Statistical analysis using Pearson's correlation showed there was no significant linear correlation between testosterone level and the total number of errors made in reaching acquisition criterion in gonadally male mice ($r = -0.089$, $n = 21$, n.s.).

Figure 6.3.8g

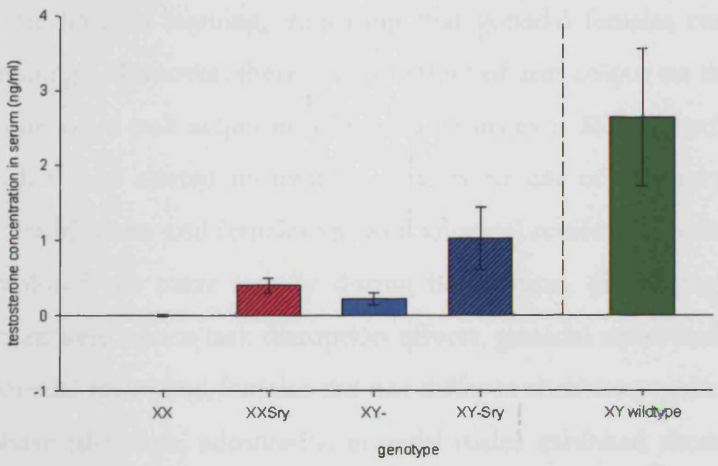


Figure 6.3.8g Testosterone level from blood serum for the five genotype groups, measured by ELISA. Data are presented as mean values ± SEM.

Figure 6.3.8h

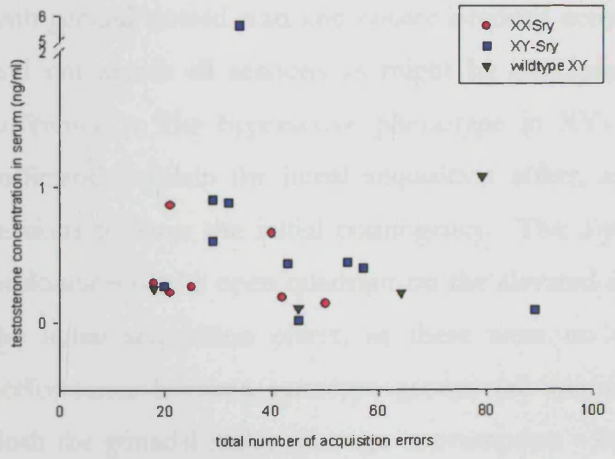


Figure 6.3.8h Correlation between total number of errors committed in achieving acquisition criterion and testosterone level.

6.4 Discussion

The purpose of this chapter was to examine *Sry*-dependent and *Sry*-independent effects on a cognitive task. The task used in the present study was a simple visual non-spatial discrimination learning task with reversal, which assayed a subject's ability to make stimulus-reinforcer associations and, following reversal, a subject's ability to inhibit prepotent responding and create novel stimulus-reinforcer associations which were the opposite of that previously acquired. There was some prior evidence from several species that gonadal hormones and/or sex-linked genes could influence one or more aspects of these psychologies.

The main finding in this chapter from the FCG model was that gonadal males (XX*Sry* and XY-*Sry*) acquired the initial stimulus-reinforcer associations with fewer errors than gonadal females (XX and XY-); this behavioural difference was particularly marked between sessions 9 and 14. Importantly, both groups began at the same level of performance, and eventually reached the same level of learning, indicating that gonadal females could learn the task if given sufficient training. Moreover, there was no effect of arm colour on this phase of the task. I argue that the more rapid task acquisition in gonadal males is likely to reflect enhanced learning in this group, rather than altered motivation, anxiety or use of olfactory cues for the following reasons: (i) gonadal males and females showed an equal reinforcer preference, (ii) gonadal males and females explored the maze equally during habituation, (iii) during odour probe manipulations, whilst there were minor task disruption effects, gonadal males and females performed equivalently, (iv) gonadal males and females did not differ in their start and choice latencies across the acquisition phase (although, admittedly, gonadal males exhibited shorter latencies to collect the reinforcer than gonadal females) and (v) the effect was particularly apparent from sessions 9-14 (coinciding with general slowed start and choice latencies across all groups, presumably as learning occurs), and not across all sessions as might be anticipated if it was due to more basic behavioural differences. The hyperactive phenotype in XY- animals observed in Chapter V could not sufficiently explain the initial acquisition effect, as both XX and XY- animals required more sessions to learn the initial contingency. The *Sry*-dependent effect on anxiety, as indexed by exploration of the open quadrant on the elevated zero maze (Chapter V), is unlikely to underlie the initial acquisition effect, as there were no differences in maze habituation and initial performance between genotype groups (all animals performed similarly during sessions 1-8). Both the gonadal males (average consumption ~2ml on Days 5 and 6) and wildtype XY males (average consumption ~1.8ml) were shown to drink more in the reinforcer preference test than gonadal females (average consumption ~1.5ml), but yet the wildtype XY males performed

equivalently as the gonadal females, which suggests that motivational differences could not completely account for the *Sry*-dependent effect in task acquisition. This gonadal sex effect in possible enhanced learning suggests that *Sry*-dependent effect underlies the difference, i.e. gonadal hormone levels and/or *Sry* direct effects on the brain, rather than sex-linked genes other than *Sry*; as explained in the Introduction section in this chapter, testosterone has been shown to exert effect on a range of cognitive behaviours and so it would not be surprising if testosterone were to influence the acquisition of stimulus-reinforcer contingencies as found in this present study (see below).

The main measure of behavioural inhibition in the reversal learning task is 'below-chance' errors (0-50%). On this measure, there were no main effects of gonadal sex or sex chromosome complement. However, an interaction between gonadal sex and sex chromosome complement (independent of arm colour) were found, whereby XX*Sry* and XY- animals made significantly more below-chance errors than XX animals. These data suggest that (i) the presence of an *Sry* transgene with an XX karyotype leads to a higher level of perseverative errors than XX karyotype alone and (ii) in subjects with a 'female' hormonal profile, the presence of an XY karyotype leads to a greater number of below-chance errors than an XX karyotype. To explain this complex pattern of results, one might speculate that there is some interaction between *Sry* and X-linked genes that could increase perseveration.

Above-chance errors reflect the ability of an animal to make an opposite stimulus-reinforcer association to one made previously. Here, whilst no effects of sex chromosome complement were observed, I did find that gonadal males made fewer errors than gonadal females. This pattern of data resembles that seen in the acquisition phase. This data should be treated with caution as an significant effect of arm colour was observed, whereby gonadal females who were initially reinforced in the black arm and reversed into the white arm, made significantly more above-chance errors than both gonadal females reversing into the black arm, and gonadal males reversing into either the black or white arms. These effects appear to be due to a particular reluctance of the gonadal females to enter the white arm, *only* once they have been consistently reinforced in the black arm (as no gonadal male vs. gonadal female differences in reactivity to the arms were obvious in the habituation or initial acquisition). Whilst this pattern of results is complex, it might suggest some female-specific interaction between the psychology and/or neurobiology associated with reacquisition and anxiety for example. The findings from the present study are inconsistent with those of Guillamon *et al.* (1986). In this previous study, no sex differences between male and female rats were seen in the acquisition of the task (whereas

the current study found an effect of gonadal sex) and quicker reversal learning in females compared to males was observed (whereas the current study found an effect of gonadal sex in total reversal errors, in which gonadal males committed fewer errors than females). These discrepancies between results might be due to species differences between studies, subtle variations in experimental designs, different acquisition criteria and the fact that the FCG mouse model do not produce normal males and so the gonadal sex effects observed in the current study might not be comparable to differences between normal males and females. However, in agreement with the current data, Davies found that wildtype MF1 male mice learnt the initial visual discrimination more rapidly than their wildtype female counterparts (Ph.D thesis, 2003).

As an additional manipulation, wildtype XY male mice were tested alongside the FCG model. During the acquisition phase, these mice resembled gonadal females, and interestingly, not *Sry* transgenic males, with regard to the number of errors committed. These data hint at the possibilities that (i) *Sry* introduced as a transgene was not equivalent to the endogenous version of the gene (in that wildtype XY mice and XY-*Sry* mice seem to be behaviourally different) and (ii) *Sry* expression from a transgene (but not an endogenous gene) may somehow enhance initial learning of the discrimination. No significant effects of genotype between the wildtype XY mice and the FCG mice (including XX females) on measures of behavioural inhibition (below-chance errors) or reacquisition (above-chance errors) were detected.

Importantly, the findings described above were not confounded by the use of other strategies to solve the task (e.g. spatial or olfactory cues). The goal arms were pseudorandomly switched in their location to avoid use of spatial strategies, and were also cleaned thoroughly after each subject to minimise the use of olfactory cues. Odour 'probe' trials were carried out to investigate any use of olfactory cues, and the extent of the effect of the probe trial was such that any olfactory guided behaviour was likely to be minimal. An advantage of the FCG model is that the four genotypes were generated at similar frequency within a single litter, and so one can minimise maternal-offspring and litter effects on behaviour, and prenatal and postnatal environment was well controlled for the FCG animals. However, the wildtype XY male mice were generated using a separate cross. Whilst care was taken to minimise any confounding effects (e.g. mothers with a uniform X chromosome were used to breed both FCG animals and wildtype males), there remained the possibility of maternal-offspring and litter effects, and differences in prenatal and postnatal environment, between the FCG model and wildtype XY males. A possible additional confound was the fact that the origin of the Y chromosome was different between males from the FCG mouse model and wildtype XY males (129 and MF1 origin respectively); there has been

evidence indicating that there are strain differences in the Y chromosome that can lead to behavioural differences, notably sexual behaviour (Canastar *et al.*, 2008) and aggression (Miczek *et al.*, 2001), but to my knowledge, there has been no direct comparison between different Y chromosomes from the 129 and MF1 strains and their effects on cognition. However, on the majority of behavioural measures in this chapter (milk preference, habituation to the maze, below-chance and above-chance errors), XY males performed equivalently to XY-*Sry* males suggesting no gross effects of generating-cross or Y chromosome origin. Moreover, all five genotype groups underwent the same husbandry procedures and were tested in parallel.

The *Sry*-dependent effect on acquisition (and above-chance errors, although note caveats above with regard to arm colour), whereby FCG gonadal males outperformed FCG gonadal females, could be due to gonadal hormone effects, or to direct *Sry* effects on the brain. It was already shown in Chapter V that blood plasma testosterone levels are higher in FCG gonadal males than females; therefore, based on the FCG data alone, one could argue that testosterone influences acquisition performance, perhaps through organisational and/or activational effects on brain regions implicated in initial learning of the discrimination e.g. prelimbic region of medial prefrontal cortex (Dalley *et al.*, 2004; Tran-Tu-Yen *et al.*, 2009; DeVito *et al.*, 2010), cingulate cortex (Bussey *et al.*, 1996; Ward *et al.*, 1999), hippocampus (McDonald *et al.*, 2007) and amygdala (Cador *et al.*, 1989). However, while the gonadal FCG males displayed higher testosterone levels and superior performance in the acquisition phase than gonadal FCG females, wildtype XY males had even higher testosterone levels than gonadal FCG males (**Figure 6.3.8g**), and yet, the performance of wildtype XY males was on par with that of gonadal FCG females. This suggests that there was no simple relationship between testosterone levels and enhanced learning in the acquisition phase of the visual discrimination task. Indeed, statistical analysis of the correlation between testosterone level and acquisition errors (**Figure 6.3.8h**) did not detect a significant linear relationship between the two variables.

With regard to the below-chance errors data, it appears plausible that there exists some complex, and as yet, undefined, interaction between the effects of *Sry* (either acting directly or indirectly) and sex chromosome complement. In terms of putative mechanisms, there is at least one X-linked gene (*MAOA*, monoamine oxidase A) for which Sry is thought to act as a transcriptional activator (Wu *et al.*, 2009). MAOA, along with MAOB, are isoforms of MAO (monoamine oxidase); MAOA, located on Xp11.23, is a key modulatory enzyme in brain biochemistry and catalyses deamination of monoamine neurotransmitters such as serotonin, adrenaline and dopamine (Shih *et al.*, 1999). Dysfunction in MAOA has been linked with a range of

neuropsychiatric disorders, such as depression (Thase & Denko, 2008), generalised anxiety disorder (Tadic *et al.*, 2003), autism (Chugani, 2002) and attention deficit hyperactivity disorder (ADHD; Gizer *et al.*, 2009); additionally it has been linked with posttraumatic stress disorder, Parkinson's disease and aggressive behaviour (Shih *et al.*, 1999).

Interestingly, some of these disorders implicated are sexual dimorphic in their prevalence and symptoms; for example, autism and ADHD are more prevalent in males than females, and females with major depressive disorders display impaired emotion processing relative to unaffected females, whereas male sufferers are not impaired compared to unaffected males (Wu *et al.*, 2009). MAOA has been consistently associated with effects on cognitive function (including behavioural inhibition) in a variety of species; for example, Fletcher and Davies (1990) found injecting a MAOA inhibitor into the dorsal raphe of rats increased their feeding in a dose-dependent fashion. The authors argued that the changes induced in serotonergic neurons in the dorsal raphe by the MAOA inhibitor might explain why animals were increasingly less capable to inhibit their feeding; indeed serotonin has been implicated in behavioural inhibition (Soubrie, 1986; Crockett *et al.*, 2009). The two gene variants of MAOA, high-activity and low-activity, have been associated with differential inhibitory control and impulsivity in normal humans in functional magnetic resonance imaging (fMRI) studies (Passamonti *et al.*, 2006; Passamonti *et al.*, 2008). Paaver and colleagues (2007) found low platelet MAO activity was associated with higher levels of impulsivity in normal adolescents, and MAO A and MAOB activity has been linked with learned helplessness in a rat study (Schulz *et al.*, 2010). There is also preliminary evidence that MAOA might be associated with IQ and general intelligence (Yu *et al.*, 2005). The fact that XY- mice appear to perseverate more than XX mice could be explained by increased *Xlr3b* expression in the former group (Davies *et al.*, 2005a); one might speculate that the effects of *Xlr3b* could be attenuated, or even reversed, in the presence of *Sry* (as XY-*Sry* mice tend to make fewer below-chance errors than XX*Sry* mice). Both *Maoa* and *Xlr3b* are expressed in the regions of the brain mediating the ability to inhibit prepotent responses, notably the orbitofrontal cortex (Meyer-Lindenberg *et al.*, 2006; Buckholtz *et al.*, 2008).

In this experimental chapter, the FCG animals and wildtype XY male mice were subject to a simple two-choice visual non-spatial discrimination task with reversal learning; the main finding was that within the FCG mouse model, gonadal males acquired the initial stimulus-reinforcer contingencies quicker (i.e. with fewer errors) than gonadal females. The behaviour of wildtype XY males, particularly in their performance on acquisition phase, was found to be similar to that of gonadal females, and not gonadal males, from the FCG cross; given that testosterone levels of

wildtype XY males were higher than FCG gonadal males and females, this would suggest that there was no simple relationship between testosterone levels and acquisition of the initial stimulus-reinforcer contingencies, and direct *Sry* effects on the brain might contribute to the observed *Sry*-dependent effect on task acquisition (see Chapter VII).

6.5 Summary

- There was no difference in reinforcer preference over water between the FCG mice; however, gonadal males were found to drink significantly more total fluid than gonadal females.

- In maze habituation, there was a significant gonadal sex × sex chromosome complement interaction on ratios of duration spent in each arm and of total number of entries.

- During acquisition phase, FCG gonadal males were significantly quicker to acquire the initial stimulus-reinforcer contingencies with fewer errors than gonadal females. The performance of the gonadal males and females differed significantly during sessions 9 to 14.

- There was a significant interaction between gonadal sex and sex chromosome complement on below-chance errors (indexing perseveration); pairwise comparisons showed that XX*Sry* and XY- animals made significantly more below-chance errors than XX animals.

- FCG gonadal males committed significantly fewer above-chance errors (indexing reacquisition of reversed contingencies) following reversal than gonadal females, although this result may have been confounded by arm colour preference.

- Wildtype XY males were found (i) to have higher levels of serum testosterone than males from the FCG model and (ii) to perform similarly to FCG gonadal females rather than gonadal males on initial acquisition of the visual discrimination task. There was no significant correlation between testosterone and number of acquisition errors, suggesting that there was no simple relationship between serum testosterone levels and enhanced learning of the initial contingencies displayed in transgenic *Sry* males.

Chapter VII

Sry brain gene expression and correlation with behaviour in Four Core Genotypes (FCG) Mouse Model

7.1 Introduction

A main finding in Chapter VI was that gonadal males from the FCG model (XX*Sry* and XY-*Sry*) acquired a simple two-choice visual non-spatial discrimination more rapidly than gonadal females from the cross (XX and XY-); the former group also showed more rapid learning on the re-acquisition of the new stimulus-reinforcer contingency following reversal (indexed by above-chance errors), though this latter effect appeared to be influenced by initial reinforced arm colour. There were complex effects on perseverative responding (indexed by below-chance errors) where gonadal sex and sex chromosome complement interacted, and it was also shown that wildtype XY males (from a separate cross) performed similarly to the FCG gonadal females rather than the FCG gonadal males.

The clear result on initial learning of the task in the FCG model suggested an *Sry*-dependent effect, which, *a priori*, could be due to indirect effects of *Sry* on gonadal hormone secretion or more direct effects of *Sry* expression in the brain. In order to address whether the effects on learning were systematically related to gonadal hormone levels, in Chapter VI (see discussion section) the performance in the initial learning phase of the task (indexed by the number of errors committed in order to reach acquisition performance criterion) was correlated with previously obtained serum testosterone concentrations in all five experimental groups (XX, XX*Sry*, XY-, XY-*Sry* and wildtype XY). This analysis showed that testosterone levels were highest in wildtype males, lower (but similar) in XX*Sry* and XY-*Sry*, and very low (or undetectable) in XX and XY- groups; this suggests that there was no systematic nor simple relationship, at the group level, between testosterone levels and this component of behaviour.

This conclusion was further supported by the fact that there was a lack of correlation between the number of acquisition errors and serum testosterone level from individual animals.

In this chapter, I examined the possibility that, rather than being due to group differences in testosterone levels, the more rapid learning in the transgenic FCG gonadal males was related to levels of *Sry* expression in the brain. There is some (to my knowledge a single) precedent for brain-expressed *Sry* directly affecting aspects of behaviour in rodent models; knockdown of *Sry* expression (specifically in the substantia nigra) in rats has been shown to result in impairments in motoric function (Dewing *et al.*, 2006). In the work detailed in this final experimental chapter, *Sry* expression in two brain regions was examined; the frontal cortex, and the midbrain (encompassing the substantia nigra and ventral tegmental area). The frontal cortex was chosen because it is known to mediate a variety of higher cognitive functions, notably aspects of two-choice discriminations and reversal tasks across species (Goldman-Rakic, 1996; Bussey *et al.*, 1996; Chudasama & Robbins, 2006; Brigman & Rothblat, 2008; DeVito *et al.*, 2010). The midbrain was chosen because it is a site of relatively high *Sry* expression (and when *Sry* levels were manipulated, it resulted in behavioural effects, Dewing *et al.*, 2006); additionally, the midbrain is rich in dopaminergic neurons and dopaminergic manipulations have been shown to influence learning in a variety of contexts (Berridge & Robinson, 1998; Kruzich & Grandy, 2004; Tran *et al.*, 2005; Rinaldi *et al.*, 2007; Bach *et al.*, 2008; Hazy *et al.*, 2010). I also examined *Sry* expression in the testes as a control; firstly, because *Sry* is known to be expressed in the germ and Sertoli cells of the adult testis (Koopman *et al.*, 1990; Rossi *et al.*, 1993) and secondly, because, *a priori*, I did not anticipate the likelihood of expression in testis influencing behaviour. *Sry* expression was assayed using sensitive quantitative polymerase chain reaction (qPCR) methods, in order to be able to detect the anticipated relatively low levels in brain tissue (Lahr *et al.*, 1995; Mayer *et al.*, 2000), and then expression levels in the two brain regions and testes were correlated with performance in the acquisition phase on the visual discrimination maze task, as indexed by number of errors committed to criterion. My general hypothesis, given that there was no significant or simple correlation between testosterone levels and performance on the acquisition of the visual discrimination task, was that brain levels of *Sry* (but not *Sry* levels in testes) would, to some extent, predict initial learning of the behavioural task.

7.2 Materials and methods

7.2.1 Subjects and animal husbandry

Animals from the Four Core Genotype (FCG) mouse model (XX, XY-, XX*Sry*, XY-*Sry*) and wildtype XY male mice were culled at the end of behavioural testing, aged 24 months old. Details on general animal husbandry and handling can be found in Chapter II (**2.2**, and **2.3**), and generation cross particulars can be found in the General Introduction, Chapter II (**2.1.2** and **2.1.3**) and V (5.**2.1**). Wildtype XY animals were produced by mating wildtype MF1 males (Harlan, U.K.) with uniform X chromosome females; the production of these females was summarised in the General Introduction. The number of subjects used in this experimental chapter is summarised in **Table 7.2.1**.

Table 7.2.1i Numbers of subjects (n) used in gene expression analyses of the various regions of interest by qPCR. Numbers in parentheses refer to the total number of animals used, including those subsequently excluded from the statistical analyses (see section **7.2.6** for exclusion criteria).

Region of interest	Genotype and 'n'				
	40,XX	40,XX*Sry*	40,XY-	40,XY-*Sry*	40,XY
Frontal cortex	0 (10)	10	0 (6)	11 (12)	8 (11)
Midbrain	0 (10)	9 (10)	0 (6)	11 (12)	10 (11)
Testes	N/A	10	N/A	10 (11)	10 (11)

7.2.2 Tissue removal, dissection and storage

After culling by cervical dislocation, brain (midbrain and frontal regions) and testes were immediately removed, dissected and stored, as described in Chapter II (**2.11**). The midbrain section included regions of ventral tegmental area and substantia nigra. The frontal region comprised of prelimbic, infralimbic, cingulate and orbitofrontal cortices, together with overlying motor and premotor cortex.

7.2.3 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction, or qPCR, was used here to examine the levels of mRNA and thus, gene expression, in the regions of interest in FCG and wildtype male animals. Briefly, as detailed below, it involved the extraction of RNA from the tissue and digestion of DNA

contaminants. RNA was reverse transcribed to cDNA, and the amplification of cDNA under PCR conditions was detected using SYBR green, a dye which intercalates between newly synthesised double-stranded DNA and which fluoresces under ultraviolet light.

7.2.3.1 RNA extraction

The frozen tissue sample and 1ml of TRI reagent (Sigma-Aldrich, U.K.) were added to a Lysing Matrix D tube containing ceramic beads (beads diameter 1.4mm; MP Biomedicals). The contents were homogenised in the ribolyser machine (Hybaid Ltd., U.K.) twice at the speed setting of four, for 15 seconds, in order to avoid overheating. 200µl chloroform was added to the tube and shaken for 10 seconds. After centrifugation for 15 minutes at 4000rpm and at 4°C, the top clear supernatant was transferred to an eppendorf tube. An equal volume of ice cold isopropanol was added and left for 5 minutes, after which the tube was centrifuged for 10 minutes at 4000rpm and at 4°C. The supernatant was poured off and pellet retained. Next, 1ml 75% ethanol was added to the tube, vortexed for 10 seconds, and centrifuged for 5 minutes at 4000 rpm and 4°C. The liquid was drained off and the pellet was air dried. 50µl water was added, and the tube was put on the heating block at 50°C for 10 minutes and vortexed for 20 seconds. NanoDrop (Thermo Scientific, U.S.A.) was used to determine the concentration and quality of the RNA; the volume containing 1µg RNA was calculated.

7.2.3.2 Digestion of DNA contaminants

5µl DNAase buffer (10x) and 1µl of DNAase (Ambion, U.S.A.) were added to the tube and was put on the heating block at 37°C for 30 minutes. 5.5µl DNA inactivation agent (Ambion, U.S.A.) was added, left at room temperature for 15 minutes, and centrifuged for 1 minute at 13000rpm and at 4°C.

7.2.3.3 cDNA synthesis from RNA

1µg of DNA-free RNA, made up to 20µl with water, was added to a Sprint RT Complete – Random Hexamer tube (Clontech, U.S.A.). The cDNA synthesis involved incubation at 42°C for 60 minutes, and then termination of the reaction by heating at 70°C for 10 minutes. 80µl water was added to the 20µl mixture to make up a total of 100µl.

7.2.3.4 qPCR

The following quantities of solution were added together for the qPCR mastermix (10µl for a single sample): 7.5µl Sensimix, 1.6µl water, 0.3µl forward primers (10µM), 0.3µl reverse primers

(10μM), 0.3μl SYBR Green (double stranded DNA fluorescent dye). 5μl of cDNA mixture was added to the mastermix. The procedure was performed using a PCR Setup pipetting robot (CAS-1200, Corbett Life Science, Qiagen, U.S.A.) to ensure consistency between qPCR runs and to minimise human error in pipetting. Each sample was done in triplicate. The qPCR was run in the Rotor-Gene 6000 machine (Corbett Life Science, Qiagen, U.S.A.) under the following conditions: 95°C for 10 minutes, [95°C for 15 seconds, 60°C for 20 seconds, 72°C for 15 seconds], repeating bracketed steps a total of 40 times. Details on the primers used can be found in **Table 7.2.3i**. Three non-template controls (5μl of water added as sample, instead of cDNA) were included in each qPCR run.

Table 7.2.3i Sequences of the forward and reverse qPCR primers used in this chapter.

Gene	Primer direction	Sequence
Hprt	Forward	5'TTGCTCGAGATGTCATGAAGGA3'
	Reverse	5'AATGTAATCCAGCAGGTCAGCAA3'
18S	Forward	5'GTAACCCGTTGAACCCCATT3'
	Reverse	5'CCATCCAATCGGTAGTAGCG3'
Sry	Forward	5'AGCAGCAGCAGCAGTTCCAT3'
	Reverse	5'GTGGTGGTGGTGGTGGTCAT3'

7.2.4 Housekeeping control genes

The expression of two housekeeping genes was analysed, in order for the expression of my gene of interest, *Sry*, to be relatively quantified. These housekeeping genes are expressed ubiquitously in all samples and act as a control, against which the expression of *Sry* could be measured. The control genes used in this study were *Hprt* (hypoxanthine guanine phosphoribosyl transferase) and *18S*. *Hprt* is a gene on the X chromosome expressed in all mammalian cells, coding for an enzyme involved in purine interconversion (Stout & Caskey, 1985). *18S*, located on murine chromosome 17, encodes for 18s ribosomal RNA found in ribosomes. Both *Hprt* and *18S* are common control genes used frequently in qPCR analyses (Hprt: Mayer *et al.*, 2000; Davies *et al.*, 2005a. 18S: Bonefeld *et al.*, 2008; Relkovic, Ph.D thesis, 2009). For each sample, CT values obtained for *Hprt* and *18S* were averaged to give a single value used for normalisation.

7.2.5 Relative quantification of gene expression: $2^{-\Delta\Delta CT}$ method

The $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001) is a common method for relative quantification; this technique gives the expression of the gene of interest in terms of control gene expression (**Figure 7.2.5a**). The crossing threshold, or C_T, value is the number of cycles it takes each reaction to reach an arbitrary amount of fluorescence (VanGuilder *et al.*, 2008), and the C_T values for each sample are set at the earliest cycle possible, at the beginning of the linear phase of the reaction.

Figure 7.2.5a

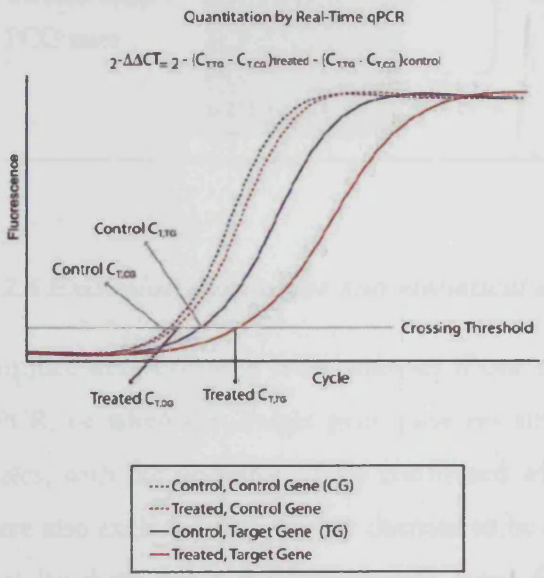


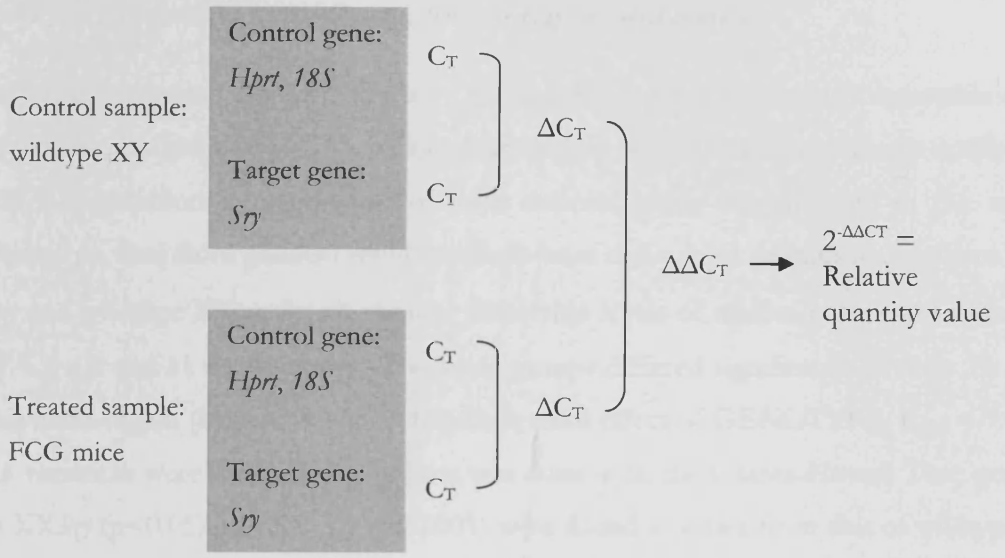
Figure 7.2.5a The $2^{-\Delta\Delta CT}$ method is used for relative quantification of the expression of a gene of interest, in terms of control gene expression, through measurements of crossing thresholds (C_T).

Diagram taken from VanGuilder *et al.*, 2008, with permission.

In the example outlined in **Figure 7.2.5a**, there are two samples (Control and Treated) and the Control and Target genes of these two samples are measured, and a C_T value is set for each of the four reactions. A ΔC_T value was obtained for Control and Treated samples by calculating the difference between the C_T values of Control and Target genes. A $\Delta\Delta C_T$ value was calculated by subtracting the ΔC_T value of the Control from the ΔC_T value of the Treated sample. The $\Delta\Delta C_T$ value is then fed into the $2^{-\Delta\Delta CT}$ equation to obtain the relative quantity value (RQ).

In the present experiment, FCG and wildtype male mice served as Treated and Control samples respectively, and *Hprt* and *18S* were the Control genes with *Sry* as the Target gene (**Figure 7.2.5b**).

Figure 7.2.5b Calculation of $\Delta\Delta C_T$.



7.2.6 Exclusion of samples and statistical analysis

Samples were excluded from analyses if one of the Control genes gave no amplification on the qPCR, or when the Target gene gave no amplification when it was expected (e.g. in gonadal males, with the presence of *Sry* confirmed with PCR). Data points (transformed values, $2^{-\Delta CT}$) were also excluded if they were deemed to be extreme outliers (extreme outliers were data points that lay three times the interquartile range from the hinges, as calculated by SPSS software). Graphs were drawn with relative quantity ($2^{-\Delta\Delta CT}$) values, in terms of gene expression of wildtype males and relevant error bars (Isles *et al.*, 2004)[20]. Statistical analyses were performed on transformed values, $2^{-\Delta CT}$, which are linear rather than exponential (as C_T values are).

Statistical analyses were performed using SPSS software (version 17, SPSS Inc., IBM, U.S.A.). Data were subject to One Way ANOVA, with factor GENOTYPE (i.e. XX*Sry*, XY-*Sry*, wildtype XY; note that data from XX and XY- gonadal females were not available as there were no detectable levels of *Sry* expression). When initial ANOVA revealed a significant effect, Tukey HSD Test, or in the cases of unequal variances, Games-Howell Test, was performed for *post hoc* comparisons. Correlational analyses were performed using the Pearson Correlation Test. For all comparisons, p values of <0.05 were regarded as significant.

[20]While calculating $\Delta\Delta C_T$, the linear error from the first ΔC_T was carried over. At the last step when one was estimating the final $2^{-\Delta\Delta CT}$, the lower and upper bound of $2^{-\Delta\Delta CT}$ would need to be calculated whilst taking into account the previously calculated linear errors, and thus, the final error bars would be asymmetrical in the $2^{-\Delta\Delta CT}$ graphs.

7.3 Results

7.3.1 Sry expression in midbrain, frontal region and testes

As expected, gonadal female animals (XX and XY-) did not show any detectable expression of *Sry* in the midbrain region (C_T values higher than 40); this was not due to confounds such as RNA degradation as expression of both control genes was detected in the same samples. Therefore, data from gonadal female animals were not subject to statistical analyses. XX*Sry*, XY-*Sry* and wildtype XY males all showed detectable levels of midbrain *Sry* expression (ΔCT values of 4.3, 4.8 and 11 respectively). The three groups differed significantly in their *Sry* expression in this brain region (**Figure 7.3.1a, left panel**; main effect of GENOTYPE, $F_{2,27} = 7.827$, $p<0.01$). As variances were unequal, *post hoc* test was done with the Games-Howell Test; gene expression in XX*Sry* ($p<0.05$) and XY-*Sry* ($p<0.001$) were found to differ from that of wildtype XY animals (approximately 50-100 times greater expression in the former groups), but XX*Sry* and XY-*Sry* expression did not differ significantly from each other.

Figure 7.3.1a

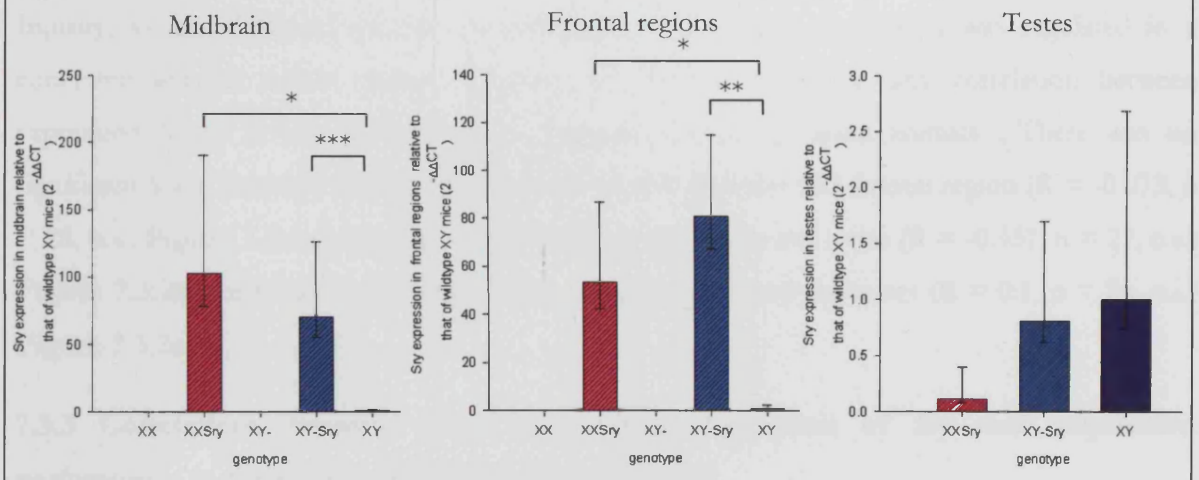


Figure 7.3.1a (left to right) Relative *Sry* gene expression in the midbrain region, frontal region and testes in FCG and wildtype XY male animals. Data are presented as relative quantity values, with upper and lower change of relative quantity errors. * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

In the frontal region, once again, as expected, there was no detectable expression of *Sry* in gonadal females, and no data from these two genotype groups were subject to statistical analysis. XX*Sry*, XY-*Sry* and wildtype XY males all showed detectable levels of frontal cortex *Sry* expression, although generally expression in this region was approximately 10 fold lower than in

the midbrain[21] (ΔCT values of 8.5, 7.9 and 14.2 respectively). Again, the three groups differed significantly in their *Sry* expression (**Figure 7.3.1a, centre panel**; main effect of GENOTYPE, $F_{2,26} = 4.318$, $p<0.05$). Games-Howell *post hoc* test revealed significant differences between XX*Sry* and wildtype XY ($p<0.05$) and between XY-*Sry* and wildtype XY ($p<0.01$) (approximately 50-100 times greater expression in *Sry* transgenic mice), but there was no difference in expression between the two transgenic groups.

Although Sry is known to be expressed and influence differentiation of the bipotential gonad around 11 to 12 days post-coitum in mice (Hiramatsu *et al.*, 2009), expression of the gene was detectable in adult XX*Sry*, XY-*Sry* and wildtype XY testes (ΔCT values of 6.7, 3.9 and 3.6 respectively). This is consistent with a previous report which found Sry expression in adult mouse testes, most likely in the germ cells (Koopman *et al.*, 1990). In contrast to the effects in the brain, the three groups did not differ significantly in their *Sry* expression in this tissue (**Figure 7.3.1a, right panel**; effect of GENOTYPE, $F_{2,29} = 1.679$, n.s.).

7.3.2 Correlations of *Sry* expression in various tissues

Initially, as an additional analysis to investigate whether *Sry* expression was regulated in a concerted manner across tissues, I examined whether there was any correlation between expression levels in the various tissues analysed within individual animals. There was no significant linear correlation between *Sry* levels in the midbrain and frontal region ($R = -0.073$, $n = 28$, n.s.; **Figure 7.3.2a**), between *Sry* levels in the midbrain and testes ($R = -0.357$, $n = 27$, n.s.; **Figure 7.3.2b**), or between *Sry* levels in the frontal region and the testes ($R = 0.1$, $n = 26$, n.s.; **Figure 7.3.2c**).

7.3.3 Correlations between brain and testes expression of *Sry* and acquisition performance in the simple visual discrimination task

The expression of *Sry* in the three tissues was correlated with the number of errors made in the acquisition phase of the visual discrimination task. There were no significant linear correlations between this behavioural measure and any of the three regions, midbrain ($R = -0.041$, $n = 19$, n.s.; **Figure 7.3.3a**), frontal region ($R = -0.095$, $n = 18$, n.s.; **Figure 7.3.3b**) or testes ($R = 0.121$, $n = 21$, n.s.; **Figure 7.3.3c**).

[21] There were approximately 3 to 4 cycle difference on average between midbrain and frontal cortex, i.e. around 2^3 and 2^4, which is around 8 to 16 fold difference.

Figure 7.3.2a

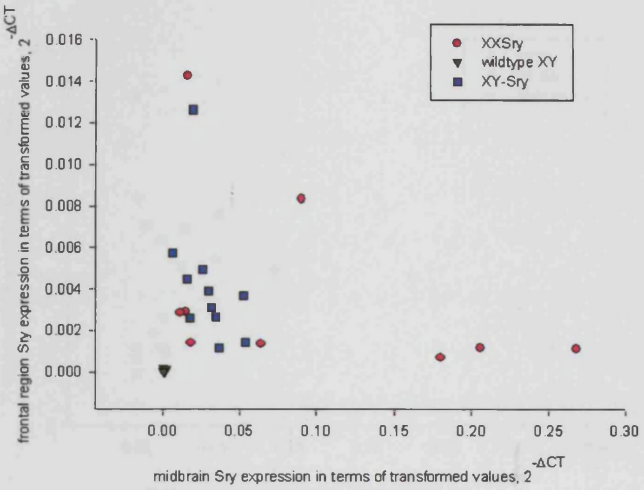


Figure 7.3.2a Correlation between *Sry* expression in frontal regions and midbrain.

Figure 7.3.2b

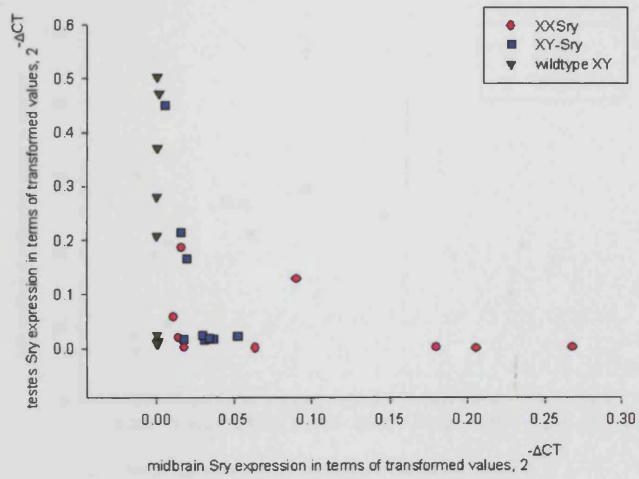


Figure 7.3.2b Correlation between *Sry* expression in testes and midbrain.

Figure 7.3.2c

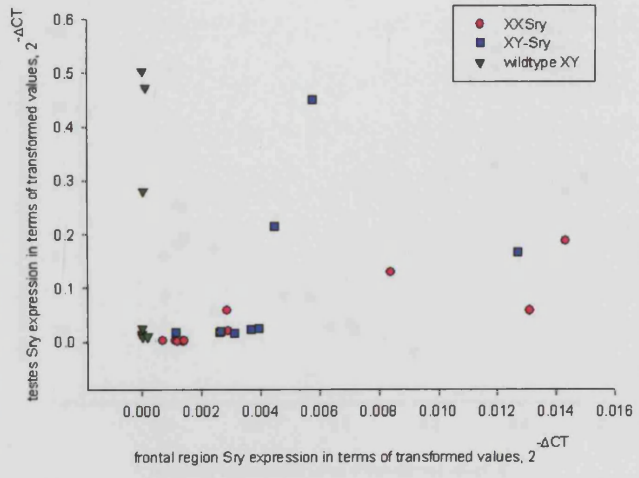


Figure 7.3.2c Correlation between *Sry* expression in testes and frontal regions.

Figure 7.3.3a

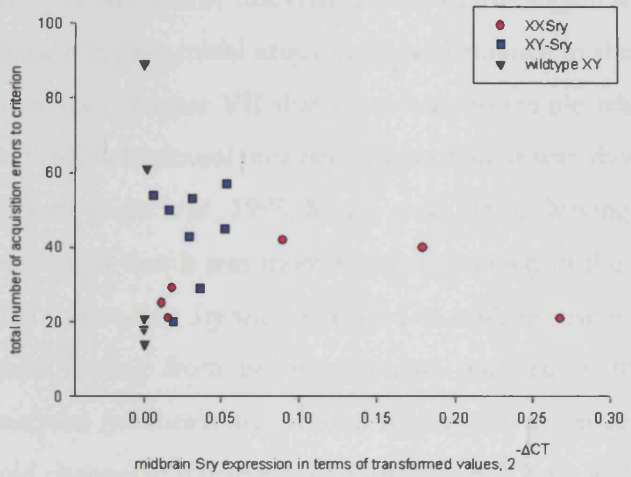


Figure 7.3.3a Correlation between acquisition performance as indexed by number of errors committed and *Sry* expression in midbrain.

Figure 7.3.3b

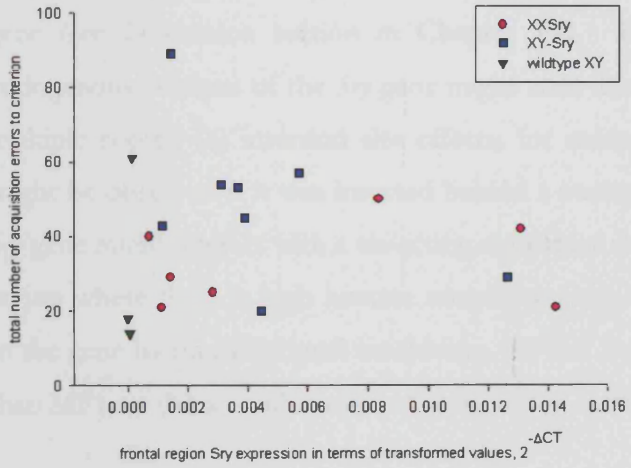


Figure 7.3.3b Correlation between acquisition performance as indexed by number of errors committed and *Sry* expression in frontal region.

Figure 7.3.3c

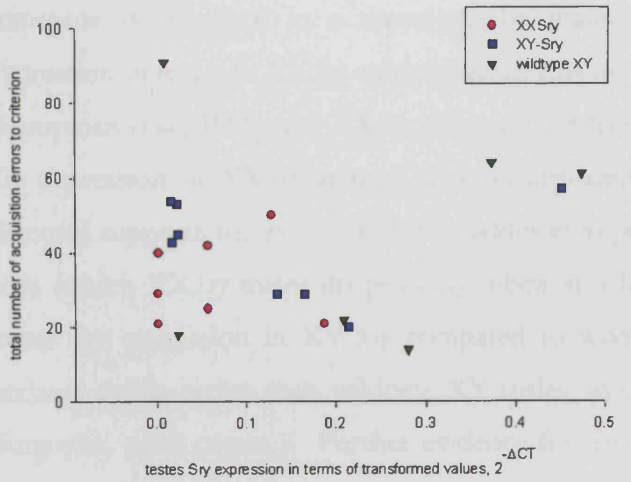


Figure 7.3.3c Correlation between acquisition performance as indexed by number of errors committed and *Sry* expression in testes.

7.4 Discussion

The primary aim of this chapter was to investigate whether *Sry* expression in the brain predicted, at least in part, initial acquisition performance in the visual discrimination task, given that it was shown in Chapter VII that there was no simple relationship between serum testosterone levels and this behavioural measure. First of all, it was shown that, consistent with previous findings in rodents (Lahr *et al.*, 1995; Mayer *et al.*, 2000; Dewing *et al.*, 2006), *Sry* was expressed in the mouse brain, and that it was more highly expressed in the midbrain than in the frontal region. It was also shown that *Sry* was expressed in adult mouse testes (Koopman *et al.*, 1990). Secondly, a key main finding from the experiments outlined in this chapter was that in both brain regions analysed (midbrain and frontal region), *Sry* expression was much higher (approximately 50-100 fold change) in transgenic FCG mice (i.e. XX*Sry* and XY-*Sry*) than in wildtype XY males.

These data provide direct confirmation of my previous speculation from behavioural data that the *Sry* transgene is not expressed equivalently in comparison to the endogenous version of the gene (see Discussion section in Chapter VI). The difference between the transgenic and endogenous versions of the *Sry* gene might arise due to (i) the *Sry* transgene has been inserted in multiple copies, (ii) insertion site effects; for example, higher expression of the *Sry* transgene might be observed if it was inserted behind a strong ubiquitous promoter and/or enhancer, the *Sry* gene might interact with a cis-acting regulatory element, or the *Sry* gene might be inserted in a region where there is high histone acetylation/low histone methylation, leading to easier access to the gene by transcriptional machinery, (iii) the *Sry* transgene being derived from a strain other than MF1, or (iv) a combination of these above factors.

Interestingly, in contrast to brain *Sry* expression, there were no significant differences in *Sry* expression in testes between transgenic FCG and wildtype XY animals; this suggests the *Sry* transgene is regulated in a tissue-specific manner. There was a tendency towards lower expression in testes in XX*Sry* animals; given that *Sry* is expressed in the germ cells of adult testes (Koopman *et al.*, 1990) and XX*Sry* males do not have spermatids (Hacker *et al.*, 1995), this lower *Sry* expression in XX*Sry* animals was unsurprising. The fact that some *Sry* expression was detected supports the evidence that in addition to germ cells, *Sry* is also expressed in the Sertoli cells (which XX*Sry* males do possess), albeit at a lower level (Rossi *et al.*, 1993). The slightly lower *Sry* expression in XY-*Sry* compared to wildtype XY males might suggest that XY-*Sry* become sterile earlier than wildtype XY males, in concordance to previous observations (Paul Burgoyne, pers. comm.). Further evidence for tissue-specific regulation of *Sry* expression was

obtained through correlational analyses between tissues, such that within individual mice, there was no obvious linear correlation between expression in the frontal region, midbrain and testes.

Overall, looking at the data in a more groupwise fashion, the general pattern of *Sry* expression in the brain (but not in testes) mirrors that of the acquisition errors (see Chapter VI), in that animals with the highest levels of brain *Sry* expression (i.e. XX*Sry* and XY-*Sry*) acquired the initial stimulus-reinforcer contingency with a significantly lower number of errors than animals with low (wildtype XY), or absent (XX and XY-), levels of brain *Sry* (**Figure 7.4a**).

Figure 7.4a

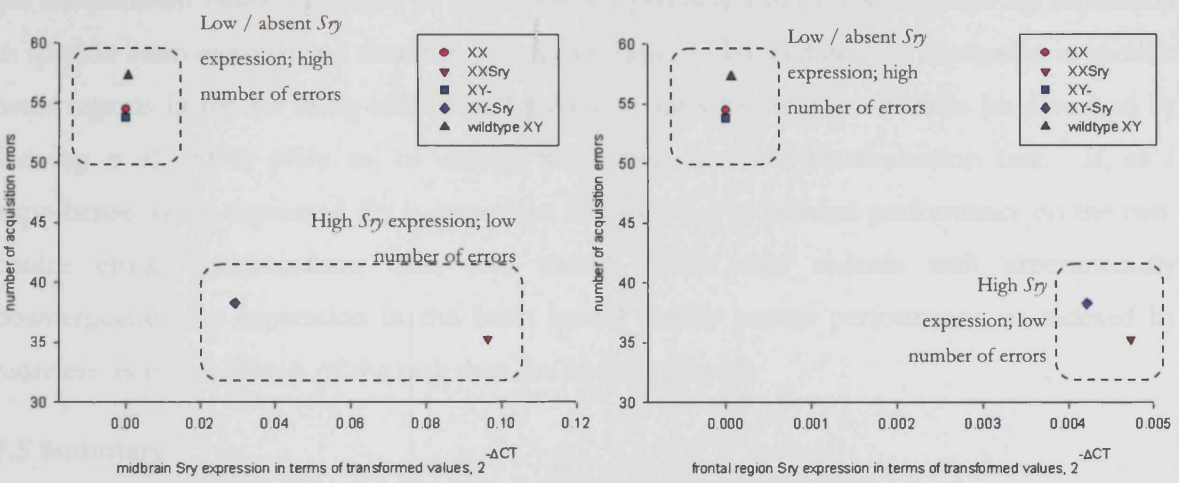


Figure 7.4a Graph showing the number of errors committed in order to reach acquisition performance criterion in relation to *Sry* expression in midbrain and frontal region. Values are presented as genotype group means.

However, these groupwise data need to be considered cautiously, as when the relationship between brain *Sry* expression in both regions and acquisition performance was examined at an individual level across the three gonadal male groups (XX*Sry*, XY-*Sry* and wildtype XY; i.e. correlating an individual's *Sry* expression with its corresponding acquisition errors), no significant linear correlations were found between either midbrain or frontal region *Sry* expression and behavioural index of acquisition performance. The lack of correlation might be due to the relatively small numbers of mice used in the analyses (hence low power to detect an effect) or, more likely, to the fact that there is no obvious linear relationship between *Sry* brain expression levels and behavioural performance and/or intermediary neurobiology (e.g. various monoaminergic functions). There was also the issue of dissection crudity, in particular, in the dissection of the frontal regions, which might encompass too many specific brain regions for a clear and significant correlation to emerge.

Given the current data obtained, I would suggest that it is possible for brain expression of *Sry* to play a role in acquisition performance in the visual discrimination task. To examine this idea further, ideally one would like to test mice with a conditional knockout of *Sry*, whereby *Sry* deletion is restricted to adult animals and relevant specific brain regions (possibly via a cre-lox recombination mechanism), on the visual discrimination task; however, these mice would be technically very difficult to generate. An alternative may be to downregulate *Sry* in the brain during performance on the visual discrimination task; in mice, this may be achieved by infusion of siRNA into the ventricles (e.g. as previously described for knockdown of the serotonin transporter gene expression in the mouse brain; Thakker *et al.*, 2005). However, the small size of the mouse brain limits the ability to infuse siRNA precisely and to downregulate *Sry* expression in specific brain regions, and therefore, it may be easier to knockdown *Sry* expression in specific brain regions in the rat using siRNA technology or antisense oligonucleotides (as described by Dewing *et al.*, 2006) prior to, or during, an analogous visual discrimination task. If, as I hypothesise, brain-expressed *Sry* is important in mediating acquisition performance on the two-choice visual discrimination task, one would predict that rodents with experimentally downregulated *Sry* expression in the brain would display poorer performance, as indexed by more errors in acquisition of the task than the control animals.

7.5 Summary

- *Sry* expression was successfully detected in the brain using qPCR.
- *Sry* expression in the midbrain and in the frontal region was significantly higher in XX*Sry* and XY-*Sry* than in wildtype XY males, with no significant differences between the two transgenic males. Approximately 50-100 times greater expression was found in XX*Sry* and XY-*Sry* relative to wildtype XY males. *Sry* expression was found to be higher in midbrain than frontal region, consistent with previous reports.
- There were no significant differences in *Sry* expression in the adult testes between XX*Sry*, XY-*Sry* and wildtype XY males.
- There were no significant correlations between *Sry* expressions in any of the three regions examined; together, the *Sry* expression data suggest tissue-specific regulation in *Sry* transgene expression.
- Whilst there was a groupwise correlation between *Sry* brain expression and total number of acquisition errors to criterion in the two-way visual discrimination task (transgenic males

with high expression of brain *Sry* made fewer errors, whilst the remaining groups with low levels of brain *Sry* expression made more errors), there were no significant correlations between *Sry* expression in the three tissues analysed and behavioural performance across individual subjects.

Chapter VIII

General Discussion

8.1 Aims of the thesis

This thesis aimed to investigate the influence of genes on the sex chromosomes on brain and behaviour using two mouse models (the XO mouse and the Four Core Genotypes (FCG) model). These models allowed the dissociation between sex-linked genetic mechanisms that could influence brain function and behaviour indirectly (i.e. via gonadal differentiation initiated by *Sry* and subsequent hormone secretion) or directly (i.e. via male-limited Y-linked gene expression, X-monosomy effects or X-linked genomic imprinting). It was hoped that the data generated in the course of the thesis would provide insights into mechanisms underlying sexual differentiation of the brain in rodents, and possibly also in humans.

8.2 Main findings

In Chapter IV, I used a behavioural task taxing biconditional rule learning and response conflict, using the XO mouse. This novel task was adapted for use in mice from a similar task used previously in rats (Haddon & Killcross, 2005, 2006a, 2006b; Marquis *et al.*, 2007); lesions of the prefrontal cortex have been shown to impair the ability to perform the response conflict aspect of the task in both rats (Haddon & Killcross, 2005, 2006a) and mice (Amy Reichelt, pers. comm.). An X-monosomy effect was observed on the acquisition of two separate biconditional discriminations (visual and auditory), such that 39,$X^{m}O$ and 39,$X^{p}O$ animals acquired the biconditional discriminations with significantly more sessions than 40,XX females; importantly, data from Chapter III, which examined the basic physiological and behavioural phenotypes of the XO mouse, suggested that this finding was unlikely to be due to non-specific factors, such as gross sensory deficits or anxiety in XO mice. All karyotype groups showed high levels of discrimination on the congruent trials, and worse performance on the incongruent trials (due to the increased difficulty and response interference), as expected. 39,$X^{m}O$ and 39,$X^{p}O$ animals were capable of completing both congruent and incongruent trials, but 40,XX animals could only perform correctly on congruent, but not incongruent, trials.

A second main finding from this thesis (Chapter V) was the observation that XY- mice (i.e. gonadally female mice with a male karyotype and a lack of the endogenous *Sry* gene) were considerably more hyperactive and habituated to the novel environment (i.e. Day One) at a slower rate, than their littermates from the FCG model (XX*Sry*, XY-*Sry* and XX) as assayed by a locomotor activity paradigm. Whilst there is no simple explanation for this finding, it is possible that one or more Y-linked genes interact with *Sry* and modulate locomotor activity and habituation. A third main finding (Chapter V) was that FCG mice transgenic for *Sry* (XX*Sry* and XY-*Sry*) tended to spend more time exploring the open quadrants of the elevated zero maze than non-transgenic littermate mice (XX and XY-), possibly reflecting a lower level of anxiety in the former groups. A fourth main finding of the thesis (Chapter VI) was that FCG mice transgenic for *Sry* (XX*Sry* and XY-*Sry*) acquired a two-way visual discrimination more rapidly than non-transgenic littermate mice (XX and XY-). In reversal learning following acquisition of this discrimination, the FCG mice transgenic for *Sry* (XX*Sry* and XY-*Sry*) also showed fewer above-chance errors (reflecting reacquisition) than non-transgenic littermate mice (XX and XY-), but there were no clear genotype effects in below-chance errors (reflecting perseveration). Again, importantly, these behavioural effects were not due to general non-specific differences between animals of the FCG model (Chapter V). The *Sry*-dependent effects on anxiety and learning (Chapters V and VI) could potentially be explained by *Sry* effects on gonadal differentiation and subsequent testosterone secretion, or by *Sry* expression in the brain; data on circulating testosterone levels and *Sry* brain expression from Chapter VII indicated that the latter possibility was more likely for the learning effects. On the other hand, testosterone levels were more likely to underlie the effect in anxiety; a significant correlation between testosterone levels and anxiety in individual animals (four genotypes from FCG model and wildtype XY males) has been shown ($p=0.023$).

8.3 Limitations of the models

The main findings described above, whilst interesting, should be viewed with some degree of caution and the limitations of the two model systems utilised would need to be taken into account.

Whilst the X-monosomy effect on acquisition of the biconditional discrimination task is unlikely to be confounded by the 39,$X^{P}O$ and 39,$X^{m}O$ mice being generated in separate crosses (a major caveat with this model), there are a number of other potential factors which may possibly influence this finding. First of all, the number of 40,XX mice that acquired the biconditional discriminations was relatively low ($n = 7$), and there was some variability in their data; therefore,

it is possible that this 'X-monosomy effect' is a Type I error. The low numbers of 40,XX mice (n = 5) for the response conflict phase of the task could result in insufficient power, leading to a Type II error (i.e. detection of a difference between 39,XO and 40,XX animals when in fact there was none). However, 40,XX mice performed equivalently to *Paf* heterozygote females on these measures (acquisition of biconditional discriminations and response conflict, data not shown), so the possibility of Type I and Type II errors is less unlikely. Ideally, the task would be repeated with larger numbers of 40,XX mice to reduce the possibility of Type I and II errors. A second potential problem with using female mice is that of oestrus status effects on behaviour. Again, I do not believe that oestrus influenced the main finding on acquisition of the biconditional task in that 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX mice had similar oestrus cycle lengths (Chapter III) and all groups contained an approximately equivalent spread of oestrus cycle stages during initial acquisition of biconditional discriminations and response conflict. Finally, there was some variability both between and within animals across testing sessions, and animals were unable to learn the task to a very high level of discrimination (>0.8); these phenomena could be due to inherent mouse biology, to strain effects, to sex effects (whereby males outperform females), or to task limitations. There is some preliminary evidence that male C57BL/6 mice might be able to acquire the biconditional discriminations with more ease; however, that particular study utilised a different performance criterion (Amy Reichelt, pers. comms). I believe that the biconditional discrimination and response conflict tasks for mouse still require some modification in the paradigm to ensure that it can capture the mouse psychology accurately.

One limitation of the FCG model is that wildtype XY male mice cannot be generated from the FCG cross and requires a separate generating cross. Therefore, it is possible that the behavioural difference between males of the FCG and wildtype XY males on initial acquisition of the two-way visual discrimination task (Chapter VI) is simply a cross-effect; steps were taken to try and negate this confound (e.g. using wildtype XY males with a uniform X chromosome). A second major limitation of this cross is that the *Sry* transgene is expressed far more highly than the endogenous version of the gene in the adult brain (but apparently not in the testes; Chapter VII); higher transgenic than endogenous *Sry* expression has also been observed at the embryonic genital ridge during development (De Vries *et al.*, 2002). One implication of this is that XY-*Sry* and XY mice cannot be regarded as equivalent. A third limitation of the data presented using this model, is that only testosterone levels in aged mice was examined (i.e. not during behavioural testing); hence, the possibility that testosterone levels correlate to a greater extent with behaviour during performance of the task than those measured here in this instance cannot be completely excluded. In previous studies using the FCG model, mice have been gonadectomised (Quinn *et*

al., 2007; Gioiosa *et al.*, 2008; Chen *et al.*, 2009; Barker *et al.*, 2010) and in some studies, given testosterone-releasing implants to ensure a consistent background hormonal milieu against which any genetic effects may be expressed (Gatewood *et al.*, 2006). I opted not to undertake gonadectomy for the following reasons: (i) I wanted to examine genetic effects on behaviour as mediated by hormones and to correlate behavioural performance with hormone levels, (ii) due to the possibility of subgroups of FCG model mice reacting differently to the surgery (anaesthesia, pain etc.) and (iii) due to the inherent limitation of gonadectomy in that it can only normalise hormone levels in postnatal mice, and not in *in utero* subjects (when hormones exert a major organisational effect).

8.4 Potential neurobiological mechanisms

Assuming the main behavioural findings of this thesis can be replicated, it will be interesting to investigate their neurobiological underpinnings.

The putative X-monosomy effect on learning in the biconditional discrimination task is presumably due to haploinsufficiency for one or more genes on the mouse X chromosome which escape XCI; to date there are 13 murine genes which have been identified to escape XCI (Yang *et al.*, 2010). Whilst there are currently no data in rodents suggesting which brain regions are important in acquisition of the biconditional discriminations specifically (e.g. lesion studies), a recent neuroanatomical analysis, conducted by myself and colleagues from Centro Nacional de Biotecnologia, Spain (Drs Marta Nieto and Beatriz Cubelos), has shown that spine density on cortical glutamatergic neurons may be sensitive to X-monosomy effects (reduced density in XO mice, **Figure 8.4a**). The extent to which genes escaping XCI influence spine density, and how spine density might then influence learning of the biconditional discriminations are questions that may be addressed in future work. There is evidence that mutant mice with aberrant NMDA receptors display decreased dendritic spine density in CA1 hippocampal neurons and impaired corticohippocampal learning and memory (Brigman *et al.*, 2010); however, there has been no study to my knowledge that links spine density with the biconditional discrimination task specifically. In Chapter IV, I suggested tentatively that differences in caudate nucleus structure and/or function may underlie the X-monosomy effect on acquisition of the biconditional response; additionally, there is evidence for aberrant caudate nucleus function (Haberecht *et al.*, 2001) and volume (Murphy *et al.*, 1993; Cutter *et al.*, 2006) in TS females relative to controls. Therefore, it may be worthwhile comparing this brain region in more detail in 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX mice.

The XO mouse may be regarded as a model for aspects of brain development and behaviour in individuals with TS, which is most commonly caused by X-monosomy (Lynn & Davies, 2007). My present data suggest that TS individuals may be slower to acquire biconditional discriminations than normal 46,XX females, and may show other deficits in cognitive functions that are dependent upon the same, yet unknown, brain regions as those mediating the biconditional discrimination acquisition process. Moreover, it is possible that TS subjects, like XO mice, show altered cortical neuron morphology, which may partially explain their characteristic behavioural phenotype. There is evidence that aberrant neural pruning might be present in TS females, which could contribute to abnormal spine density (Kesler *et al.*, 2003; Rae *et al.*, 2004).

Figure 8.4a

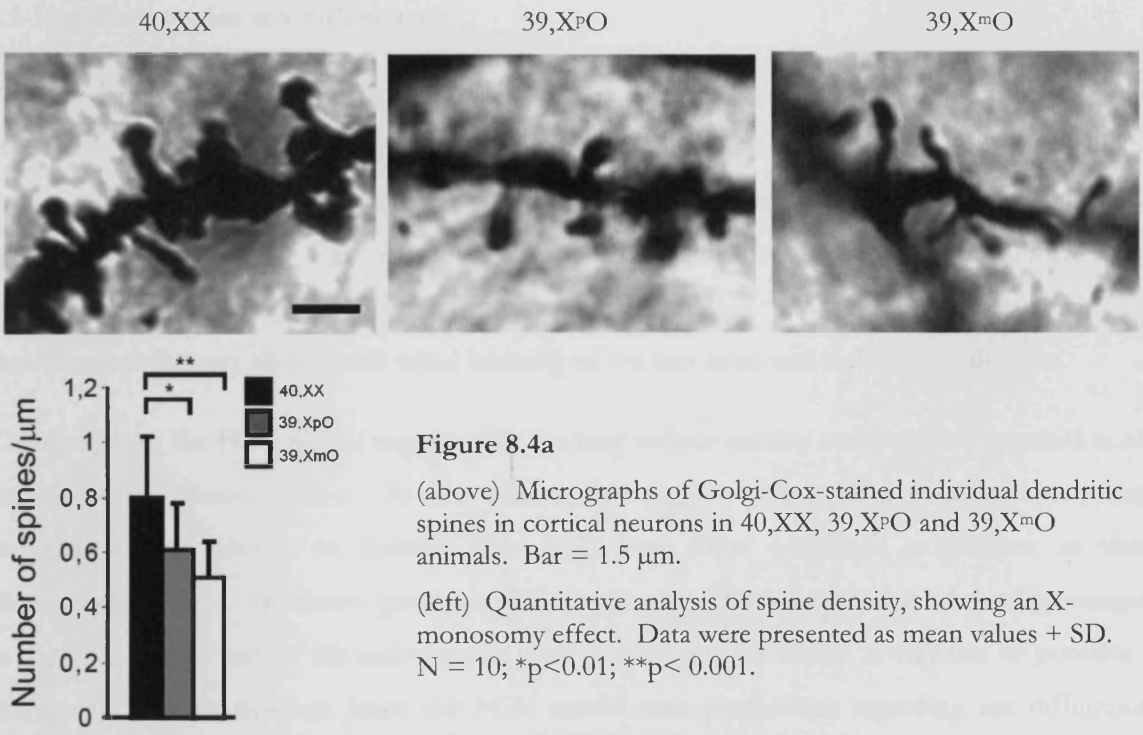


Figure 8.4a

(above) Micrographs of Golgi-Cox-stained individual dendritic spines in cortical neurons in 40,XX, 39,X^{P}O and 39,X^{m}O animals. Bar = 1.5 µm.

(left) Quantitative analysis of spine density, showing an X-monosomy effect. Data were presented as mean values + SD. N = 10; *p<0.01; **p< 0.001.

The *Sry*-dependent effect on initial acquisition of the visual discrimination task with FCG model seems most likely to be due to a direct effect of brain-expressed *Sry*. This may act via a defined mechanism to influence behaviour, for example, through modulation of the dopaminergic and/or monoamine system via transcription activational effects on TH (Milsted *et al.*, 2004) and MAOA (Wu *et al.*, 2009) respectively, or through some, as yet, unspecified mechanism. There is also evidence that *Sry* might modulate the expression of many autosomal genes (Wijchers *et al.*, 2010). Furthermore, the tissue-specific regulation observed in this thesis (i.e. the increased

expression in the *Sry* transgene was restricted to brain and not observed in the testes) and the developmental time point and tissue -dependent switch from circular (embryonic) to linear (adult) *Sry* mRNA transcripts in the brain (Capel *et al.*, 1993; Jeske *et al.*, 1995; Mayer *et al.*, 2000) lend support to the notion that brain-expressed *Sry* could play an important role in brain function. The number of TH-ir neurons in the anteroventral periventricular nucleus of the preoptic region has been shown to be lower in transgenic *Sry* animals (XX*Sry* and XY-*Sry*), compared to XX and XY- gonadal females and wildtype XY males (De Vries *et al.*, 2002); this pattern of results is similar to that obtained from the initial acquisition of the visual discrimination task, giving more support to the idea that TH might mediate the *Sry*-dependent acquisition effect. If the *Sry*-dependent effects on learning are mediated via effects on TH and/or MAOA levels, I might expect pharmacological modulation of the dopamine axis to attenuate or exacerbate this effect.

8.5 Implications for sex differences

The main finding from the XO mouse model suggests the presence of one or more X-linked genes that escape XCI enhanced learning of the biconditional discrimination task. Genes that escape XCI may, theoretically at least, be more highly expressed in female than in male tissues (assuming no functionally equivalent Y homologue, and no interaction with gonadal hormones); this X-linked gene dosage effect contributes to sex differences. Hence, I may tentatively predict that female mice may show more rapid learning of the biconditional task than male mice.

The data from the FCG model suggest that *Sry* may reduce anxiety and improve acquisition of a two-way visual discrimination. At first glance, this suggests that normal male mice may show reduced anxiety relative to female mice, and may show enhanced acquisition in visual discrimination tasks. However, given the different levels of brain expression of the *Sry* transgene in the FCG model and of the endogenous gene in wildtype XY males, it may not be possible to extrapolate directly findings from the FCG model into predictions regarding sex differences. Whilst it was indeed shown that wildtype XY males displayed reduced fear reactivity than XX females from the FCG model in a possibly testosterone-related fashion, as indexed by exploration of the open quadrants of the elevated zero maze, in Chapter VI it was found that wildtype XY males and XX females from the FCG model performed equivalently on the initial acquisition of the two-way visual discrimination. The findings with regard to the FCG mouse model cast some questions on the validity of the model. *A priori*, the FCG mouse model is a means to dissociate between effects of sex chromosome complement (i.e. XX vs. XY) and effects of *Sry* (i.e. gonadal type male vs. gonadal type female, and/or brain effects of *Sry*). However, the extremely high level of *Sry* brain expression found in the transgenic animals would

suggest that the model is not looking at gonadal type male vs. gonadal type females (i.e. gonadal hormone dogma), nor normal brain effects of *Sry*, but is in fact testing the effects of *supraphysiological* levels of transgenic *Sry* (at least in the brain) against sex chromosome complement effects. In addition, there might be some yet unknown interactions between the (supraphysiological) levels of *Sry* and sex chromosome complement, and therefore, when there is an effect of sex chromosome complement found in the analysis, one has to consider whether the (supraphysiological) level of *Sry* has influenced the sex chromosome complement effect to a significant degree. Whilst the FCG model might be useful in being able to predict sex differences in the normal range to some extent, given the supraphysiological levels of *Sry* transgene expression, it might have more application in clarifying the neurobiological mechanisms underlying behavioural abnormalities (e.g. ADHD) in conditions such as 47,XYY in which *Sry* over-expression might occur. Risk genes for ADHD have been mapped to the short arm of the Y chromosome (where *Sry* is located), with evidence that children with ADHD are more likely to inherit risk factors from their father rather than mother, and ADHD has been shown to be more common in males with 47,XYY karyotype (Milligan *et al.*, 2008).

8.6 Future work

The thesis has generated some exciting data which are worthy of follow-up analyses. One important avenue for future work will be to investigate the neurobiological mechanisms that differentiate 39,XO mice from 40,XX females, and notably the possible mechanisms underlying the X-monosomy learning effect seen in 39,XO mice on the biconditional discrimination task. This could be done through a number of approaches, guided by parallel lesion studies in wildtype female MF1 mice: (i) the expression of immediate-early genes (e.g. *c-Fos*) throughout the brain could be compared between 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX mice learning the biconditional discrimination task, and 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX mice not undertaking the task, as an index of neural activity; I anticipate that expression would be different in 39,$X^{m}O$ and 39,$X^{p}O$ in regions mediating the learning process, (ii) *in vivo* microdialysis in brain regions of interest in 39,$X^{m}O$, 39,$X^{p}O$ and 40,XX mice; given the neuroanatomical data above, it may be particularly interesting to examine synaptic levels of glutamate and to perform NMDA receptor-targeted pharmacological manipulations and, (iii) to see whether the learning deficits observed in 39,$X^{m}O$ and 39,$X^{p}O$ can be rescued through genetic means, for example, by introducing transgenes for candidate X-linked loci, or by examining the behaviour of XY^{*X} mice (essentially 39,XO mice with a small number of additional genes including the X-inactivation escapees *Sts* and *Mid1*; Davies *et al.*, 2007). In addition to further neuroanatomical studies, it would be useful to

perform more behavioural analyses using the XO mouse model. These could focus on 'learning' tasks to see whether the X-monosomy deficit in the biconditional discrimination task is generalisable to other tasks, such as those known to be highly dependent upon frontal cortex function (e.g. working memory tasks such as delayed non-matching to position; Chudasama & Muir, 1997), or tasks that assay psychologies which are known to be altered in Turner syndrome (e.g. visuospatial ability).

A second important avenue for future research will be to determine more directly whether brain-expressed *Sry* can influence behaviour and cognition directly (notably, the acquisition of a two-way visual discrimination). This may be done by knockdown of the gene in brain regions known to underlie the particular psychology of interest, or by using antisense oligonucleotides or siRNA. Alternatively, it may be possible, though technically difficult, to generate mice which express or lack the *Sry* transgene only in certain brain regions or at certain developmental time-points. These manipulations could be performed in association with pharmacological manipulations of the dopamine system to determine the extent to which the effects of brain-expressed *Sry* are mediated via this neurochemical system. Further work in the FCG model might look at aspects of cognition that are known to be sexually dimorphic in healthy individuals and/or individuals affected by various disorders. One particular aspect of cognition which may be of particular interest is attention, given the preponderance of males with attention deficits, and the increased susceptibility of XYY males to ADHD (Milligan *et al.*, 2008). I have tested the same FCG mice on the 5-choice serial reaction time task, which assesses visuospatial attentional functioning, impulsivity and motivation (Humby *et al.*, 1999; Robbins, 2002), and found that gonadal males (XX*Sry* and XY-*Sry*) showed significantly more premature responses than gonadal females (XX and XY-) under the 'long inter-trial interval (ITI)' (changing ITIs from 5 seconds at baseline, to 5, 6, 7, 8 seconds) and the 'short stimulus duration (SD)' (changing SDs from 0.8 seconds at baseline, to 0.8, 0.5, 0.3, 0.1 seconds), manipulations. The performance of wildtype XY males was more similar to gonadal females than gonadal males, recapitulating the pattern of results found in the initial acquisition of the visual discrimination task. This *Sry*-dependent effect on impulsivity, which appears to be exacerbated by higher attentional demands, suggests that *Sry* (brain-expressed *Sry* rather than indirect effects via gonadal hormones, given the performance of wildtype XY males) might mediate some aspects of impulsivity directly and help explain the higher incidence of ADHD in males.

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