Sexual Differentiation of the Brain: Investigating the role of *Sry* using animal models

by
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List of abbreviations

5-HT	serotonin
5-HIAA	5-Hydroxyindoleacetic acid
aCSF	artificial cerebrospinal fluid
AD	Alzheimer's disease
ADHD	Attention-Deficit Hyperactivity Disorder
AH	anterior hypothalamus
AMH	anti-mullerian hormone
ANCOVA	analysis of covariance
ANOVA	analysis of variance
AR	androgen receptor
AVPV	antero-ventral paraventricular nucleus
BD	bipolar disorder
BNST	bed nucleus of the stria terminalis
CAH	congenital adrenal hyperplasia
cDNA	complementary DNA
CS	conditioned stimulus
CSF	cerebrospinal fluid
Ct	cycle threshold
DA	dopamine
df	degrees of freedom
DHEA	dehydroepiandrosterone
DHT	dihydrotestosterone
DMN	dorsal medial nucleus
DNA	deoxyribonucleid acid
DOPAC	3,4-dihydroxyphenylacetic acid
dpc	days *post coitum*
E	estradiol
EEG	electroengephalogram
ELISA	enzyme-linked immunosorbent assay
EPM	elevated plus maze
ER	oestrogen receptor

FCG	four core genotype
Fosl1	Fos related antigen 1
GABA	γ-Aminobutyric acid
GAD	generalised anxiety disorder
Gat-1	GABA transporter 1
GDX	gonadectomised
HMG	high mobility group
Hprt	hypoxanthine guanine phosphoribosyl transferase
ITI	inter-trial interval
KS	Klinefelter's syndrome
LSD	least significant difference
MA	medial nucleus of amygdala
MAOA	monoamine oxidase A
MPH	methylphenidate
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
NAc	nucleus accumbens
NACWO	Named Animal Care and Welfare Officer
NRX	non-recombining region of the X
NRY	non-recombining region of the Y
ODN	oligonucleotide
ORFC	orbitofrontal cortex
PAR	pseudoautosomal region
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
Pdyn	prodynorphin
PET	positron emission tomography
PFC	prefrontal cortex
Plp	proteolipid protein
POE	parent-of-origin effect(s)
PR-ir	progesterone immunoreactive
PS	phosphoriothioate

PTSD	post traumatic stress disorder
RNA	ribonucleic acid
qPCR	quantitative (real-time) polymerase chain reaction
SAP	stretched attend posture
SCR	skin conductance response
SDN-POA	sexually dimorphic nucleus of the preoptic area
SEM	standard error of the mean
SF1	steroidogenic factor 1
SN	substantia nigra
SNB	spinal nucleus of bulbocavernosus
Sox9	SRY box containing gene 9
Sry	sex-determining region of Y
SSt	somatostatin
Sts	steroid sulphatase
TDF	testis determining factor
TH	tyrosine hydroxylase
Tph2	tryptophane hydroxylase 2
TS	Turner syndrome
US	unconditioned stimulus
VMN	ventral medial nucleus
vmPFC	ventromedial prefrontal cortex
VTA	ventral tegmental area

Summary

Recent findings have challenged the dogma that gonadal hormones are the sole factor mediating sexual differentiation of the mammalian brain, and have highlighted the importance of sex-linked genes in this process, either independently, or in combination with, gonadal hormones. Using two rodent models, this thesis investigated the roles of the Y-linked (male-specific) gene *Sry* and other sex chromosome-linked genes on emotional behaviour. Using an established murine 'four core genotype' (FCG) model an attempt was made to dissociate, '*Sry*-dependent effects' (due to direct effects of *Sry* brain expression, or to downstream hormonal factors) and effects due to 'sex chromosome complement' (i.e. sex-linked genes other than *Sry*). The mouse work was augmented by the development of a novel rat *Sry* antisense 'knockdown' model permitting investigation of the behavioural effects of direct manipulation of *Sry* expressed in discrete brain areas.

The main finding from the FCG model was a *Sry*-dependent effect on anxiety-related and exploratory behaviours, whereby transgenic *Sry* expression appeared to act to reduce the former and promote the latter. A physiological role for an anxiolytic (anxiety reducing) function of *Sry* was supported by sexually dimorphic differences in anxiety behaviours between normal male (expressing *Sry* endogenously from the Y chromosome) and female mice; as predicted from the FCG model, male mice were less anxious than females. In apparent contradiction of the data obtained in mice, acute antisense mediated reductions of *Sry* expression in rat ventral tegmental area, were also anxiolytic, an effect that may be partially mediated via expression changes in the known *Sry*-regulated gene *Fosl1*. Possible explanations for the differences in the direction of effects on behaviour in the mouse and rat models are discussed. Overall, the data highlight a role for *Sry*-mediated effects on emotional behaviour.

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Chapter I

General Introduction

The objective of this thesis was to investigate the biological mechanisms underlying sex differences in behaviour, and in particular to compare and contrast the contributions of gonadal sex with other mechanisms related to sex chromosome complement. There is a substantial body of evidence showing that in many mammalian species, including humans, males and females differ in aspects of physiology and behaviour; the first part of this introductory chapter summarises studies examining sex differences in humans, both in healthy individuals and those affected by brain disorders. The second part of the chapter reviews the literature on sexual dimorphisms in behaviour and brain function observed in rodents, the animal model used in the present work. A third section develops current ideas concerning the biological basis of sexual differentiation of the brain, including evidence that challenges a sole influence of gonadal hormones; this section also provides a detailed account of the main rodent models to be used in the experimental work and lists the overall aims of the thesis. Throughout the thesis, the term "sex" is used instead of "gender", as the studies presented herein focus on the biological factors that shape the different characteristics observed between males and females, rather than the societal.

1.1 Sex differences in brain and behaviour: evidence from human studies

1.1.1 Sex differences in healthy individuals

There is an abundance of studies in healthy individuals investigating potential sex differences across many aspects of behaviour, cognition and brain structure/function. For example, in psychological tests past studies have revealed an apparent female advantage in verbal fluency and episodic memory tasks. Specifically, women appear to outperform men in lexical and category word generation tasks (where participants are asked to write as many words as they can imagine beginning with a specific

letter), as well as in word recall and face recognition tasks (Herlitz, Nilsson, & Backman, 1997; Weiss, Kemmlera, Deisenhammer, Fleischhacker, & Delazer, 2003; Weiss et al., 2006). In contrast, men tend to perform better at certain spatial-navigational problems, such as mental rotation and virtual maze tasks (developed to mirror rodent spatial memory processes) (Driscolla, Hamilton, Yeo, Brooks, & Sutherland, 2005; Kimura, 1996; Maguire, Burgess, & O' Keefe, 1999; Weiss, et al., 2003). Sex differences have also been reported in brain function and structure. For example, it has been claimed that women's brains have proportionally more grey matter and are more symmetrical than men, while men have proportionally more white matter and cerebrospinal fluid (CSF) (Gur et al., 1999). In terms of overall brain volumes, women have larger volume in most frontal and medial paralimbic regions, whereas men have larger frontomedial cortex, hypothalamus, amygdala, and angular gyrus volumes (Goldstein et al., 2001).

However, it must be stressed that the findings of brain and behavioural differences between men and women are not without controversy. More recent studies and meta-analyses have questioned the validity of many of the aforementioned results (Rahman, Bakare, & Serinsu, 2011; Sommer, Aleman, Bouma, & Kahn, 2004; Wallentin, 2009). The lesson seems to be that, research into sex differences in human psychology and behaviour is a particularly complex area with many factors influencing subjects' performance (notably the experimental design and hormonal fluctuations across the female menstrual cycle). Nonetheless, some data does seem to hold up and Table 1.1.1i summarises sex differences that are observed consistently across a range of psychological and behavioural domains in healthy humans.

Table 1.1.1i: Sex differences in normal human psychology and behaviour

Measure	Details of sex difference	Reference
Fear and anxiety	Evidence indicating that women score higher in anxiety-related factors.	(McLean & Anderson, 2009)
Neural correlates of emotion processing	Females tend to be more emotionally perceptive and reactive, while males tend to exhibit a greater degree of emotional regulation. Differences on brain activation are also discussed.	(Whittle, Yucel, Yap, & Allen, 2011)
Learning and memory	Review on four main aspects: spatial, verbal, autobiographical and emotional memory. Female advantage has been shown not only in verbal, but also in some spatial and episodic memory tasks.	(Andreano & Cahill, 2009)
Neuroimaging methods	Sex differences on brain structure, function and chemistry (e.g. men have greater total brain volume and white matter, while women have greater grey matter; women have higher serotoninergic and dopaminergic function than men).	(Cosgrove, Mazure, & Staley, 2007)

1.1.2 Sex differences in human brain disorders

In addition to studies conducted on healthy individuals, there is a large literature on sex differences in risk for, and patterns of presentation within, a number of brain disorders, both neuropsychiatric and neurodegenerative. Whilst it is beyond the scope of this chapter to discuss all relevant findings, it should be mentioned that men, notably, are more prone to 'neurodevelopmental' disorders such as ADHD, autism and antisocial personality disorder, whilst women are more likely to be diagnosed with affective disorders, such as anxiety and depression (Holden, 2005). Sexual dimorphisms are also evident in other common mental disorders such as schizophrenia (Grossman, Harrow, Rosen, Faull, & Strauss, 2008).Table 1.1.2i

summarises some key sex differences in brain disorders with respect to risk/prevalence, course, possible underlying neurobiology changes, and response to therapy.

Table 1.1.2.i: Sex differences in human brain disorders

Disorder	Measure	Details of sex difference	Reference
General psychopathology	Prevalence	Male susceptibility to early-onset developmental disorders; female susceptibility to adolescent-onset emotional disorders.	(Rutter, Caspi, & Moffitt, 2003)
Unipolar depression	Prevalence and symptoms	Prevalence for women is twice that of men; women report more vegetative and atypical symptoms, anxiety and anger than men. In self-report measures, women report more severe depression.	(Scheibe, Preuschhof, Cristi, & Bagby, 2003)
Unipolar depression	Treatment response	Women responded better and faster to sertraline, whereas men to imipramine.	(Kornstein et al., 2000)
Unipolar depression	Neurobiology	Women were shown to have smaller amygdala, while men smaller left inferior anterior cingulate. No volumetric differences were reported in hippocampus and orbitofrontal cortex (ORFC).	(Hastings, Parsey, Oquendo, Arango, & Mann, 2004)
Schizophrenia	Symptoms and recovery	In a longitudinal study on patients' sample, women demonstrated a lower rate of psychotic activity, better recovery, and global	(Grossman, et al., 2008)

		functioning than men.	
Schizophrenia	Symptoms, onset, course, neurobiology	Disease onset is later (~ 3-5 years) for women than men; women report more affective and positive symptoms, whereas men more negative; some studies have reported ventricular enlargement in women, but not men, and larger planum temporale in men; oestrogen might be neuroprotective for women; post-menopausal women need higher dose of antipsychotic medication compared to men.	(Hafner, 2003)
Alzheimer's disease (AD)	Prevalence	Women are at a higher risk of developing the disorder than men.	(Gao, Hendrie, Hall, & Hui, 1998)
Alzheimer's disease (AD)	Pathology, clinical manifestation	Women had more AD pathology (neurofibrillary tangles, neuritic plaques) than men. Furthermore, there is a stronger relation between AD pathology and clinical disease, and a greater cognitive deterioration (episodic, semantic, working memory, perceptual speed, visuospatial ability) as AD progresses, than in men.	(Barnes et al., 2005)
ADHD	Symptoms	ADHD girls show lower levels of hyperactivity, fewer conduct problems, lower rates of	(Gaub & Carlson, 1997)

		externalising behaviour, and greater intellectual impairment compared to ADHD boys.	
ADHD	Treatment response	Sex differences in response to methylphenidate (MPH). Females displayed superior MHP response during the early part of the day and faster decline than males.	(Sonuga-Barke et al., 2007)
ADHD	Electrophysiology (EEG), and psychophysiology (skin conductance response/SCR)	Male adolescents with ADHD displayed an enhanced global EEG theta activity, while females showed a reduced change in SCR during resting condition.	(Hermens, Kohn, Clarke, Gordon, & Williams, 2005)

1.2 Experimental studies of sex differences in rodents

Mice and rats constitute two of the most widely studied species in investigations of sex differences as they are relatively easy to breed and manipulate experimentally. Importantly, rodent models allow a detailed analysis of behaviour followed by subsequent easy access to brain tissue for biochemical and molecular determinations. Another advantage of rodent models is their amenability to experimental genetic modification, especially in the case of mice, a feature which was exploited in this thesis. Rodent models also have the potential to provide insights into the mechanisms underlying sex differences in humans, with the usual important caveats regarding cross-species validity. The following section will focus mainly on sex differences observed in the behaviour and brain structure/function of 'wild type' non-genetically modified animals (see later for work on genetically engineered models).

1.2.1 Sex differences in rodent behaviour

Male and female rodents have been shown to differ across many psychological and behavioural domains, from basic behaviours such as food and water consumption, to complex motor and cognitive functions. In general, male rodents weigh more and therefore consume more food than female rodents (Bell & Zucker, 1971b; Koos Slob & Van Der Werff Ten Bosch, 1975b). Females, on the other hand, have been shown to be more active in many behavioural paradigms (Koos Slob, Huizer, & Van Der Werff Ten Bosch, 1986; Lightfoot, Turner, Daves, Vordermark, & Kleeberger, 2004).

Table 1.2.1i summarises some of the most commonly reported behavioural sexual dimorphisms observed in rodent model research. Prominent sex differences can be found in emotion-related behavioural assays, such as Elevated Plus Maze and Open Field arena, two behavioural paradigms used extensively in this thesis. In the first task, rodents are free to explore a plus maze, which is comprised of two open, exposed arms, and two closed arms protected with high walls. Essentially, the more/less time the animal spends in the exposed compartments is used as an index of 'anxiety': more exploration of the open arms, less anxious; less exploration more anxious (Wall & Messier, 2001). The Open Field task is based on the natural tendency shown by rodents to display thigmotaxis (i.e. when moving staying close to the walls of an arena). Thus, animals that move away from the walls and spend time in the central part of the arena are regarded as being relatively less anxious (Simon, Dupuis, & Costentin, 1994). As females have been reported to spend more time exploring the open arms of the Elevated Plus Maze, and the centre of the Open Field, this might be taken as evidence for females, under these specific circumstances, being less anxious than males (for references see Table 1.2.1i).

Sex differences in learning mechanisms have also been documented and could, arguably, be relevant to the pathogenesis of sex-biased disorders characterised by aberrant learning such as addiction, post-traumatic stress disorder, ADHD. Recently, Dalla and Shors (2009) reviewed the rodent literature and concluded that, as in humans, sex differences in learning are complex, not always replicated between laboratories, and may interact with additional factors such as early-life stress and hormonal milieu. Hence, in rodents, females tend to show better learning of the classical eyeblink paradigm, where the animal has to learn the association between a white noise (conditioned stimulus; CS) and an aversive stimulation of the eyelid

(unconditioned stimulus; US). However, in other variants of aversive conditioning of stimuli, such as fear conditioning paradigms, where animals associate a cue or context (CS) with an aversive stimulus, such as footshock (US), and learn to respond (by freezing or expressing an enhanced startle reflex) in anticipation of the footshock, males appear to learn the contingencies more rapidly than females. Sex differences have also been reported in operant conditioning assays, where unlike the above Pavlovian learning paradigms the animal has to make an overt response (e.g. pressing a lever) in order to learn (i.e. escape an aversive stimulus, or receive food); here it has been claimed females have the advantage. Of relevance to previous work carried out in our own laboratory, males and females have also been reported to differ in learning paradigms requiring behavioural flexibility, such as reversal learning tasks. In reversal learning animals have to first acquire an initial association (i.e. stimulus A predicts availability of reinforcer, stimulus B does not), which is subsequently reversed, so that subjects have to relearn the rule, by inhibiting the old association and responding in the opposite way (i.e. stimulus B now predicts availability of reinforcer, stimulus A does not). In rats, there is evidence that females were quicker to learn the new association compared to males (Guillamon, Valencia, Cales, & Segovia, 1986), while murine data from our laboratory suggested an opposite pattern (P. M. Y. Lynn, 2010) .

Sex differences in learning and memory in rodent models can be subtle and often interact with task requirements and other factors such as stress levels. For example, in general, females are better in object recognition tasks, where they have to identify the presence of a novel object amongst ones to which they have previously been habituated. However, males perform better, if a spatial component is added to this task, so that subjects also have to remember the location of the objects (Sutcliffe, Marshall, & Neill, 2007). The Morris Water Maze constitutes a commonly used spatial memory task, where animals have to locate an invisible platform located in a circular pool filled with opaque liquid (R. Morris, 1984). When subjects have to locate the platform based on the PLACE strategy (wherein the location of the platform was fixed) males performed better than females. If, however, training is performed using different strategies, such as CUE (wherein the location of the platform changes in each trial, but the platform is visible by using a cue) or EGO (wherein subjects learn the location of the platform in association with their starting position; e.g. always turn right), then females perform equal to males (Blokland, Rutten, & Prickaerts, 2006). Female performance is enhanced in spatial tasks, such as object

location and radial arm maze (maze with eight arms, where animals have to make correct entries and remember which arms they visit) when stress levels are raised, while male rats perform worse, thus reversing the sex difference observed in control, non-stressed animals (Bowman, Beck, & Luinea, 2003).

Table 1.2.1i: Behavioural sexual dimorphisms in rodents

Species	Behavioural phenotype	Behavioural task	Details on sex difference	Reference
Rats	Anxiety	Elevated plus maze (EPM)	Females spent more time in the open arms of the EPM, suggesting lower levels of anxiety	(Johnston & File, 1991)
Mice	Anxiety	EPM, open field	Females spent more time in the central area of the open field, and more time in the open arms of the EPM, denoting less anxiogenic behaviour than males.	(Gioiosa, Fissore, Ghirardelli, Parmigiani, & Palanza, 2007)
Rats, mice	Classical/operant conditioning	Review of many tasks (i.e. fear potentiated startle, lever pressing)	Males tend to outperform females on fear conditioning; females acquire faster eyeblink and operant conditioning.	(Dalla & Shors, 2009)
Rats	Working and spatial memory	Novel object recognition task	Females performed better in a standard version of the task, while male performance was superior in a spatial version.	(Sutcliffe, et al., 2007)
Rats	Spatial memory	Morris water maze	Sex differences in the use of PLACE strategy	(Blokland, et al., 2006)

			(males are better), but not of CUE or EGO (egocentric).	
Rats	Chronic stress and memory	Radial arm maze, object location task, object recognition task	Females are in general less affected by chronic stress than males, and their performance is either unaffected or improved.	(Bowman, et al., 2003)

1.2.2 Sex differences in brain structure and neurochemistry in rodents

It is evident from the above section that whilst a complex issue, it is probably safe to conclude that behavioural differences between male and female rodents do exist. Similarly, while these behavioural differences are likely the product of many factors, it is also likely that at least some of them are related to sexually dimorphic brain structure and/or neurochemistry. Male and female rodent brains differ in many respects, such as volume of specific brain regions, neuronal density, and activity of various neurotransmitter systems[1] (Ngun, Ghahramani, Sánchez, Bocklandt, & Vilain, 2011). One of the most consistent observations concerns the sexual dimorphic nucleus of the pre-optic area (SDN-POA), which is involved in male copulatory behaviour and is much larger in male than in female rats (Gorski, Gordon, Shryne, & Southam, 1978). Other nuclei showing similar pattern are the medial nucleus of the amygdala and the bed nucleus of the stria terminalis (BNST), both involved in male sexual behaviour (DeVries, 1999; Hines, Allen, & Gorski, 1992). Furthermore, in males, these two nuclei receive denser innervations of vasopressin (DeVries & Panzica, 2006), a neurochemical involved in social and parental behaviours (Goodson & Bass, 2001). In contrast, the anteroventral periventricular nucleus of the hypothalamus (AVPV), which regulates ovulatory cycles (J. A. Morris, Jordan, & Breedlove, 2004), has been found to be larger in female than in male rats (Bleier, Byne, & Siggelkow, 1982) (see also Table 1.2.2.i). Similar findings have been reported in mice, although

[1] Rat probably constitutes the most well defined species in terms of sexual dimorphisms in the brain (see following sections for issues with mouse).

with this species extra care must be taken in the interpretation of apparent sex differences as a result of strain differences (Brown, Mani, & Tobet, 1999).

In terms of neurochemistry, sex differences have been reported in several neurotransmitter systems. An intriguing observation was the sexual differentiation of GABA and glutamate neurotransmission in neonatal brain (especially hypothalamus), as males were shown to exhibit greater activity than females (McCarthy, Davis, & Mong, 1997). Sexual dimorphisms have also been reported with regards to monoaminergic pathways, which in turn could, again arguably, be related to stress, addiction, and other sex-biased, monoaminergic-related disorders (J. B. Becker, 1999; Goel & Bale, 2010); female rats have been found to have higher dopamine (DA) levels in ventromedial prefrontal cortex (vmPFC), insula, and nucleus accumbens, as well as higher serotonin (5-HT) levels in vmPFC, amygdala, dorsal hippocampus, and insular cortex. Males, in contrast, exhibit a higher metabolic rate and neurotransmission turnover, as indexed by higher DOPAC levels in the aforementioned regions, and higher 5-HIAA levels in vmPFC, amygdala, and insula (Duchesne, Dufresne, & Sullivan, 2009).

Table 1.2.2i: Sex differences in brain structure and neurochemistry

Species	Brain region/neurochemical system	Details on sex difference	Reference
Rat	AVPV (anteroventral periventricular nucleus of hypothalamus)	Larger and with higher cell density in females than males	(Bleier, et al., 1982)
Mouse (129SvEv & C57BL/6J)	POA/AH (preoptic area/anterior hypothalamus)	129SvEv: Cells were larger in female than male mice in area ventral to AC (anterior commissure); C57BL/6J: cells in the caudal area	(Brown, et al., 1999)

		were larger in male than female mice.	
Rat	Medial nucleus of amygdala (MA), bed nucleus of the stria terminalis (BNST)	Volume region is larger in males than in females.	(Hines, et al., 1992)
Rat, mouse	Vasopressin neurons in amygdala and BNST	Males have more cells and denser projections than females.	(DeVries & Panzica, 2006)
Rat	Dopamine (DA), serotonin (5-HT)	Females have higher levels of DA and 5-HT in various brain regions than males; males have higher levels of DOPAC (DA metabolite) and of 5-HIAA (5-HT metabolite).	(Duchesne, et al., 2009)
Rat	GABA and glutamate system in neonatal hypothalamus (dorsal medial nucleus/DMN, ventral medial nucleus/VMN, arcuate nucleus), and CA1	GAD (rate limiting enzyme in GABA) mRNA and glutamate binding is higher in male hypothalamic areas than in female	(McCarthy, et al., 1997)

Importantly, these sex differences in brain structure/function may not necessarily have a well-defined sexually dimorphic behavioural correlate, and in some cases, the sex difference in neurobiology may exist to ensure that males and females exhibit an equivalent behavioural phenotype (DeVries, 2004; DeVries & Sodersten, 2009). For example, the rat sexual dimorphic SDN-POA has not been shown, so far, to have an irrefutable functional significance, albeit having been related to male sexual behaviour

(DeVries, 2004). Furthermore, studies in prairie voles have suggested that the greater vasopressin innervations in the male BST and amygdala could serve to guarantee a similar parental behaviour between males and females, rather than to create a behavioural sexual dimorphism (De Vries & Boyle, 1998).

1.3 Mechanisms of sexual differentiation of brain

For many years, the classical dogma on sexual differentiation of the brain was the notion that it was triggered and maintained primarily, or even solely, by the actions of gonadal steroid hormones. This dogma has recently been challenged and accumulating evidence suggests that genes could act independently of, or interact with, gonadal hormones to play an important part in establishing sexual dimorphisms of the brain (McCarthy & Arnold, 2011). In mammalian species, including humans, the most fundamental genetic difference between males and females is their distinct sex chromosome complements. Males possess a single X chromosome (invariably inherited from their mother) and a single Y chromosome (invariably inherited from their father); thus, they possess an XY karyotype (46,XY in humans, 40,XY in rodents) (Figure 1.3a). Females, on the other hand, inherit two X chromosomes, one from each parent (karyotype 46,XX in humans and 40,XX in rodents). The X chromosome is relatively large (~155Mb in humans, and ~166Mb in mice; (Mueller et al., 2008; M. T. Ross et al., 2005)) and contains ~1500 genes, many of which are involved in brain and cognitive functions (Zechner et al., 2001). The Y chromosome, in contrast, is small (~60Mb in humans, and ~95Mb in mice; (Alfondi, 2008; Lahn, Pearson, & Jegalian, 2001)) and contains fewer genes, many of which are involved in spermatogenesis (C. E. Bishop & Mitchell, 1999; Skaletsky et al., 2003). Sex chromosomes do not recombine, apart from at sites, towards their telomeres, named pseudoautosomal regions (PARs). The rest of the genes are located in areas termed non-recombining region of the X (NRX) and non-recombining region of the Y (NRY) (Arnold, 2004).

Figure 1.3a

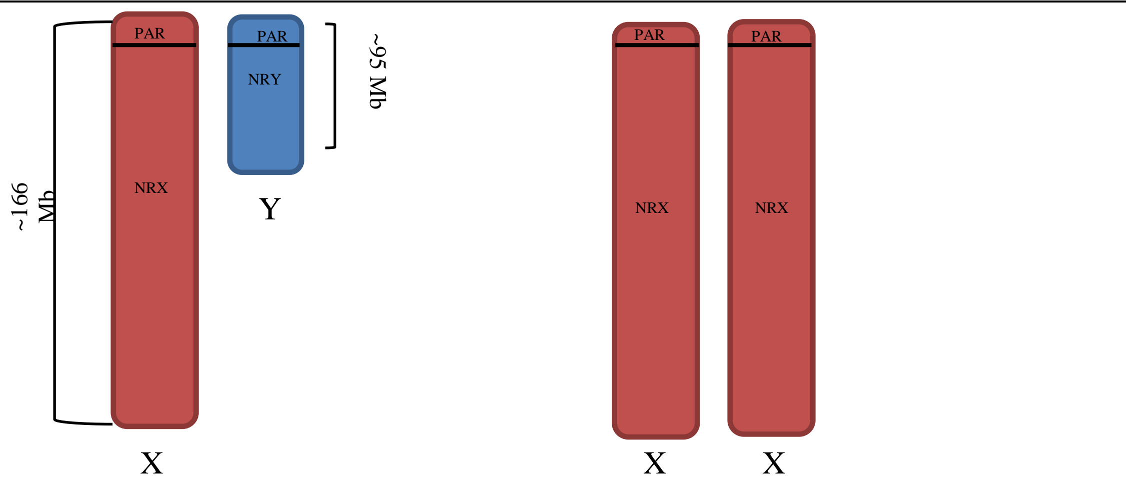

Figure 1.3a: Schematic of sex chromosomes in mice (in humans sex chromosomes have two PARs on either end of the chromosomes).

1.3.1 Influences of gonadal hormones

The classical dogma on sexual differentiation states that this process is under the control of gonadal hormones. Early experimental work by Lillie (1917) and Phoenix (1959) suggested that sex hormones (notably testosterone) have a profound effect on sexual differentiation. Lillie noted that freemartins[2] displayed a more masculine phenotype and were generally infertile, when there was mixing of placental blood between the male and female calves, which he attributed to chemicals in the blood (i.e. sex hormones), whereas Phoenix demonstrated that female guinea pigs, that were given testosterone prenatally, had a more masculine behaviour as adults. Further to that, Jost (1970) regarded the female pathway as the default route that gonads, genital tract and brain take, in the absence of the masculinising effect of gonadal hormones. Since then, an abundance of experimental data has provided support to the notion that gonadal hormones, through their organisational (early neonatal, permanent influences) and activational (reversible influences that could occur in post-natal life) effects have a profound role in masculinising and feminising the gonads and other tissues, including the brain (J. A. Morris, et al., 2004).

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[2] Freemartin is the female of two-sexed twins in cattle.

In rodents, from approximately embryonic day 15 (E15) Leydig cells initiate testosterone synthesis, which is fundamental for the development of the male reproductive tract (Wilson & Davies, 2007). Testosterone exerts its effects either directly, by acting on androgen receptors, or indirectly, through its main metabolite, estradiol, acting on oestrogen receptors. The mechanism by which conversion to estradiol occurs is via the P450 enzyme, which acts locally in the brain (Hutchison, 1997). Testosterone aromatisation to estradiol is more prominent in rodents, whereas in humans, masculinisation of the brain occurs mainly via testosterone acting directly on androgen receptors (Swaab, 2007). Female foetuses, in rodents and humans, are protected from any excessive surge of oestrogen by alpha-fetoprotein (Bakker et al., 2006; Swaab, 2007). This inhibition is lost after birth, so that females acquire higher oestrogen levels than males (Andrew, Dziadek, & Tamaokig, 1982).

Gonadal hormones can affect behaviour in many ways (Arnold, 2009b). In rodents, hormonal manipulations have been shown to affect sexual behaviour (Henley, Nunez, & Clemens, 2011), aggression (Compaan, Buijs, Pool, De Ruiter, & Koolhaas, 1993), pain perception (Craft, Mogil, & Aloisi, 2004), and learning and memory processes (Spritzer et al., 2011; Toufexis, Myers, & Davis, 2006). Genetically-modified mouse models have also highlighted a prominent role for gonadal hormones. For example, male mice lacking the aromatase gene (ArKO mice) have been shown to display deficits in mating behaviour, which are corrected when treated with oestrogen and dihydrosterone in adulthood (Bakker, Honda, Harada, & Balthazart, 2004). Furthermore, adult mice with 'testicular feminisation' mutation (Tfm), lacking functional androgen receptors (ARs), tend to display a more anxiogenic behaviour than wildtype male mice in some anxiety-related tasks (novel object and light dark box) (Zuloaga, Morris, Jordan, & Breedlove, 2008). In humans, the importance of steroid hormones becomes evident in cases of disorders of sexual development. Girls with congenital adrenal hyperplasia (CAH; a condition where the female foetus is exposed to abnormally high testosterone levels) have been shown to prefer more masculine-typical toys and activities, while as adults, they are more prone to be dissatisfied with a female sexual orientation than normal females (Hines, 2002). In terms of cognition, Hines (2002) states that studies have reported inconsistent and inconclusive findings as to whether hormonal abnormalities prenatally could alter performance in later life. A summary, necessarily selective, of research into brain and

behavioural effects of gonadal hormones in animals and humans is given in Table 1.3.1i.

Table 1.3.1i: Examples of gonadal hormonal effects on brain and behaviour (list is not exhaustive).

Species	Effect on brain/behaviour	Reference
Rat	During critical developmental period steroid hormones affect cell death, cell morphology and synaptic connectivity in various brain regions, such as SDN-POA, spinal nucleus of bulbocavernosus (SNB), BNST, AVPV, VMN, and arcuate nucleus.	(Schwarz & McCarthy, 2008)
Rat	Oestrogen manipulation affects female performance on learning and memory tasks, such as Morris water maze, and T-Maze. In water maze, females with high oestrogen levels perform poorly compared to females with lower oestrogen levels, whereas in the T-maze high oestrogen levels predispose female rats in using allocentric, and not egocentric strategies.	(Korol, 2004)
Rat/mice	Testosterone's metabolite, 3α-diol, which binds to ERβ (oestrogen receptors), enhances anxiolytic and cognitive performance in aged male rats and mice.	(Frye, Edinger, & Sumida, 2008; Osborne, Edinger, & Frye, 2009)
Mice	Dihydrosterone (DHT) treatment on the day of birth (P0) masculinised performance of female mice on an olfactory and partner preference task. Furthermore, DHT increased number of calbindin-ir neurons in female mPOA and inhibited c-Fos responses (mPOA and	(Bodo & Rissman, 2008)

	BNST) to olfactory task.	
Rats	In a T-maze reversal discrimination learning task, male orchidectomy and female testosterone treatment on P1 reversed the observed sex differences (non-treated females performed better than intact males).	(Guillamon, et al., 1986)
Humans	Females with congenital adrenal hyperplasia (CAH) tend to display more typical male behaviour (play behaviour, autistic traits, increased aggression, increased homosexual behaviour) than non-exposed females.	(Manson, 2008)

1.3.2 Sexual differentiation: beyond steroid hormones

Whilst gonadal hormones clearly play a major role in sexual differentiation of the brain and behaviour, evidence suggests that they do not act in isolation to direct/modulate this process. Research on zebrafinches provided initial data towards a new hypothesis stating that sex chromosome-linked genes could directly influence sexually dimorphic neurobiology. Specifically, male zebrafinches (ZZ chromosomes) are equipped with a courtship song circuit to attract females (ZW chromosomes), which is absent in females. This neural circuit, initially found to be instigated by estrogen secretions (Arnold, 1997), has been the focus of many researchers, who have tried, albeit unsuccessfully, to fully reverse it by hormonal manipulations; early gonadectomy does not have a profound effect in male singing pattern (Adkins-Regan & Ascenzi, 1990; Arnold, 1976). Further to that, females treated with estrogen at the first day of hatching failed to develop a fully masculinised neural song circuit, even though the hormonal treatment did have a limited effect in volume of relevant brain areas. Nevertheless, male brain regions were still larger, irrespectively of treatment (Jacobs, Grisham, & Arnold, 1995).

Additional evidence for a genetic effect was provided by the discovery of a rare gynadromorphic zebra finch. Phenotypic examination showed that the right side was typically male, with regards to plumage and gonads (dysmorphic testis), whereas the left side was typically female (plumage and presence of ovary). In terms of reproductive behaviour, the gonadromorphic finch had a fully developed male-type song pattern and was able to successfully mate with females. Analysis of whole brain tissue revealed a pattern similar to the rest of the body, as the left brain was feminised, (and contained ZW cells), whereas the right side was masculinised and contained ZZ cells. Most interestingly, the brain areas controlling the song circuit were larger in the male side than in the female. Given that both hemispheres of the brain were exposed to an equivalent hormonal milieu, it was argued that the dimorphism seen between the two was due to a

Figure 1.3.2a

Figure 1.3.2a: Image of gynadromorphic chicken (taken from Zhao et al, 2010, with permission). The left side is male, having a larger wattle, and more ZZ cells, while the right side is female and is composed mainly of ZW cells.

cell-specific, genetic control of sexual differentiation (Agate et al., 2003). Strong support for this view was also provided by more recent work from Zhao and colleagues (2010). Working with gynandromorphic chicken, these researchers showed that sex identity of somatic cells is autonomous and inherent, depending on the sex chromosomal, and not hormonal, environment (Zhao et al., 2010) (Figure 1.3.2a). It might be argued, that there is now sufficient evidence implying a direct role of sex chromosome-linked genes on sexual differentiation that future research should work

towards a modified theory, aiming to unify gonadal hormone and sex chromosomal complement influences.

1.3.3 Four core genotype (FCG) mouse model

It is evident, from data presented in the above sections that, while steroid hormones play a fundamental role in sexual differentiation, evidence from birds indicate that sex chromosome complement could also contribute to this process. Recent evidence from a novel mouse model has suggested that the same may be true in mammals. The so called "Four Core Genotype" (FCG) model was generated in order to dissociate the effects of gonadal hormones from those due to chromosomal sex complement in mammals (Arnold & Chen, 2009). The FCG mouse model, which has been employed previously in the context of brain function and behaviour (Arnold, 2009a; Arnold & Chen, 2009; DeVries et al., 2002) was used extensively in this thesis and will be described in detail later. In order to understand the complexities and subtleties of this animal model however, it is first necessary to introduce the Y-linked *Sry* gene.

1.3.4 Sry: the 'Sex-Determining gene'

In mammals, male sexual differentiation is conferred by the inheritance of the Y chromosome (Wilhelm & Koopman, 2006) . The testis determining factor (TDF) in humans, *SRY* (for Sex determining Region of Y), was first reported by Sinclair and colleagues, in a seminal Nature paper (Sinclair et al., 1990). According to their observations, human *SRY* is located at Yp53.3 and encodes a protein with a DNA-binding motif, which is present in high mobility group (HMG) proteins. In an accompanying paper, the existence of a mouse homologue, *Sry*, was noted (Yp11.2), with the gene being deleted in 40,XY mice conferring a basic female phenotype (Gubbay et al., 1990). Since this early work, considerable effort has been made to better characterise the gene and to identify the molecular mechanisms through which *Sry* acts. Briefly, data from mice indicate that *Sry* is expressed in the undifferentiated gonads during a short frame period (from embryonic day 10.5 to 12.5 in mice; from approximately day 40 of gestation in humans until adulthood). In particular, Sry acts synergistically with SF1 (steroidogenic factor 1) to upregulate *Sox9* gene expression, leading to the differentiation of Sertoli cells and testes formation (Sekido & Lovell-

Badge, 2008). *Sox9* expression levels remain high, even after *Sry* expression has ceased, initiating (with the contribution of WT1, SF1, and GATA4) the expression of anti-mullerian hormone (AMH). AMH (also known as Mullerian inhibitory substance) is pertinent for the development of the male reproductive path, as it induces regression of the mullerian tract, and subsequent formation of the uterus. Testosterone is subsequently secreted by the Leydig cells of the differentiated testes (around E15 in rats and 12-17 weeks of pregnancy in humans); (Wilson & Davies, 2007). Mutations of human *SRY* can result in sex reversal (46, XY gonadal dysgenesis) and occur mainly within the HMG motif (Cameron & Sinclair, 1997).

Given the importance of *Sry* in sex determination, it might be expected that the gene would be highly conserved across species. However, as it can be seen in Figure 1.3.4a, the *Sry* gene sequence diverges substantially, and the only conserved area is the HMG. The most striking difference could be observed between murine *Sry* and other species. Specifically, murine Sry protein has a C-terminal glutamine rich domain, which is absent in other mammals. Previous work on this domain has been inconclusive and no definite function can be attributed to the CAG trinucleotide repeat region. Truncated mutations of the *Mus musculus* Sry, lacking the C-terminal region, were indeed insufficient to cause sex reversal in XX mice, implying that the glutamine rich region is necessary for sex determination (Bowles, Berkman, Cooper, & Koopman, 1999). Conversely, it has been suggested that this region might not have any functional role, since human *SRY*, which does not have the CAG trinucleotide repeat, could also cause sex reversal in XX mice (Lovell-Badge, Canning, & Sekido, 2002). It is also of interest to note that, while most mammalian species, including humans and mouse, have one copy of *Sry*, some rodent species have multiple copies of the gene (Acosta et al., 2010; Lundrigan & Tucker, 1997). One of these species is *Rattus norvegicus*, which has been reported to have six full length copies of Sry (*Sry1*, *Sry2*, *Sry3*, *Sry3B*, *Sry3B1*, and *Sry3C*) with tissue specific differences in expression patterns (Turner et al., 2007).

Figure 1.3.4a

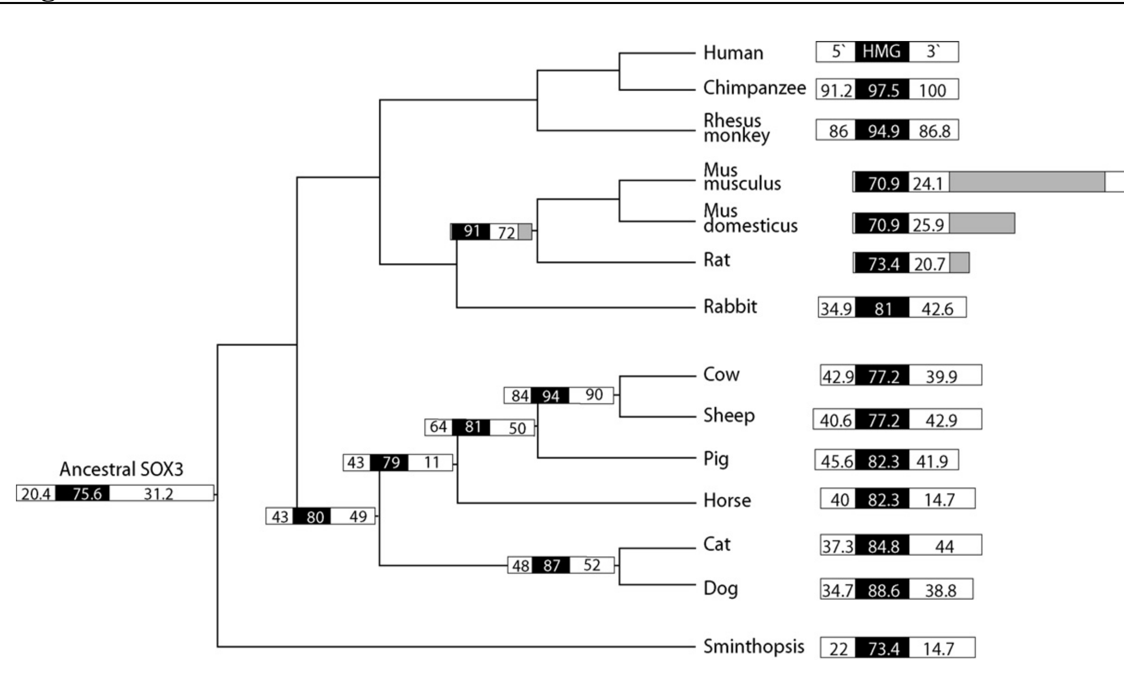


Figure 1.3.4a: SRY protein sequence among species. SOX3 constitutes the common ancestral gene. HMG box is denoted in black and flanking regions in white. C-glutamine rich terminal is in grey colour. Numbers represent percentage of homology with human SRY. Figure copied from Waters, Wallis, and Marshall Graves (2007) with permission.

1.3.5 Sry expression; gonads and beyond

Apart from its main expression in testes, *SRY* in humans is also expressed in other tissues, including heart, liver, kidney, and brain (Clepet et al., 1993; Mayer, Lahr, Swaab, Pilgrim, & Reisert, 1998). In the brain, human *SRY* is expressed in hypothalamus, frontal and temporal cortex (Mayer, et al., 1998). In mice, two different transcripts of *Sry* have been reported. Early in development (embryonic days 11-19) circular, apparently untranslated transcripts have been found, whereas in the adult brain (diecenphalon, midbrain, and cortex), linear, and potentially translatable transcripts are present (Lahr et al., 1995; Mayer, Mosler, Just, Pilgrim, & Reisert, 2000). In adult rats, *Sry* is particularly highly expressed in the midbrain (Dewing et al., 2006; Milsted et al., 2004).

The findings of persistent *Sry* expression in tissues, such as brain, means that its protein product Sry could, in theory, influence male-specific traits in two main ways. First, the protein could exert its influence either directly (via cell-autonomous

action) or indirectly (through influencing gonadal differentiation and thereafter via gonadal hormonal secretions, notably testosterone). Evidence for the former mechanism has come from work conducted by Milsted and colleagues (2004), who showed that Sry protein regulates tyrosine hydroxylase (TH, the rate-limiting enzyme in dopaminergic synthesis), by binding to its promoter and enhancing transcription. This research group has subsequently argued that Sry, by acting on TH expression, could play an important role in hypertension, a disorder with a male-bias (Turner, Ely, Prokop, & Milsted, 2011). Further *in vitro* experiments revealed that Sry could act as a transcriptional activator at the gene encoding monoamine oxidase A (Maoa), the enzyme that catalyzes monoamine metabolism (Wu, Chen, Li, Lau, & Shih, 2009). These data imply that at least one way by which Sry could shape male characteristics is through acting directly on the monoaminergic system. By extension, it might also be suggested (cautiously) that Sry could exert a direct effect on disorders associated with monoaminergic (dys)function, such as ADHD, addiction, and hypertension (Andersen & Teicher, 2000; Charchar, Tomaszewski, Strahorn, Champagne, & Dominiczak, 2003; Quinn, Hitchcott, Umeda, Arnold, & Taylor, 2007). Indeed, as previously mentioned, Sry has been implicated in hypertension and increased blood pressure, as well as in Parkinson's disease (PD) all disorders showing degrees of male-bias (Dewing, et al., 2006; Turner, Farkas, Dunmire, Ely, & Milsted, 2009).

However, it must be emphasised that, as discussed previously, there is substantial evidence for a major role for *Sry* on brain and behavioural functioning acting indirectly, that is, via its influence on gonadal differentiation, and thereafter gonadal hormonal secretions, notably testosterone. It is possible therefore, that many *Sry*-related effects on brain and behaviour are mediated by gonadal secretions of testosterone, and are not dependent on brain-expressed *Sry*. The extent to which the well-known indirect, and more recently discovered direct effects of *Sry* action, and gene effects related to sex chromosome complement (for example, X-linked genes and Y-linked genes other than *Sry,* see later), as well as their potential interactions (see later), are involved in sex differences in brain and behaviour is far from settled.

1.3.6 Details of the Four Core Genotype (FCG) model: dissociating Sry dependence from sex chromosome complement

The FCG model offers the potential to begin to tease apart effects due to *Sry* dependence from effects arising from sex chromosome complement in mice comprising of the following four genotypes: 40,XX (karyotypically and gonadally females), 40,XY- (karyotypically male, but gonadally female because of the absence of *Sry* expression), 40, XX*Sry* (karyotypically female, but gonadally male, because of transgenic *Sry* expression), and 40, XY-*Sry* (karyotypically and gonadally male where the *Sry* deletion has been compensated for by transgenic expression of *Sry*) (Figure 1.3.6a). The generation of the FCG model was made possible by two factors: the first was the characterisation of a spontaneous mutant mouse in which the *Sry* gene was deleted from the Y chromosome by Lovell-Badge and Robertson (1990). Specifically, using chimeric mice, they found a spontaneous Y-linked mutation containing the TDF area ($Y^{Tdy.m1}$ or Y-), which resulted in complete sex reversal (40, XY females). $Y^{Tdy.m1}$ is a 14kb deletion located in the short arm of the Y chromosome, which removes *Sry*, while retaining other properties of Y chromosome (Gubbay, et al., 1990; Lovell-Badge & Robertson, 1990). The second key technical advance was the successful insertion of a *Sry*-transgene onto an autosome (derived from the transgenic line C57BL/6Ei-$Y^{AKR/J}$TgN (Sry-129)2Ei) in the same mice, resulting in XY-*Sry* mice which were phenotypically male (Mahadevaiah et al., 1998). Through pairing XY-*Sry* mice with 40,XX females the distinct four genotypes of the FCG model described above may be generated.

For any given phenotype, observed differences between 40, XX and 40,XY- mice on the one hand (both gonadally female, but differing in karyotype), and between 40,XX*Sry*, and 40, XY-*Sry* on the other (both gonadally male, but differing in karyotype), would imply that the effect is due to sex-linked genes other than *Sry* (for the purposes of statistical analyses termed a 'SEX CHROMOSOME COMPLEMENT' factor effect in this thesis). If, however, there are differences between 40, XX and 40, XX*Sry* mice (possessing the same karyotype, but gonadally female and male respectively), as well as between 40, XY- and 40, XY-*Sry* mice (possessing the same karyotype but gonadally female and male respectively), then it could be argued that the effect is due to the presence (or absence) of *Sry* (factor 'SRY DEPENDENCE' in statistical analyses). *Sry*-dependent effects could, as it has already

been noted, be either direct (cell-autonomous, independent of hormonal secretions), or indirect (mediated by gonadal hormone (testosterone) secretion).

Figure 1.3.6a

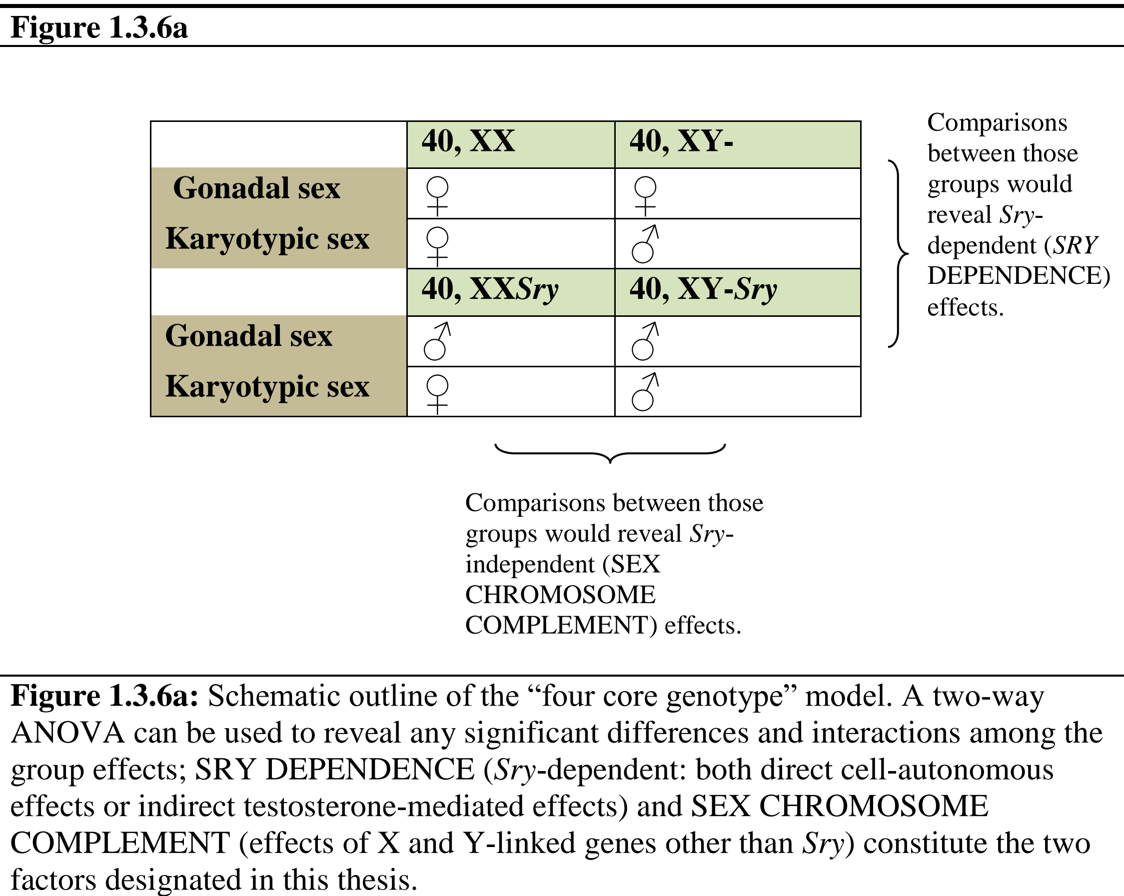


Figure 1.3.6a: Schematic outline of the "four core genotype" model. A two-way ANOVA can be used to reveal any significant differences and interactions among the group effects; SRY DEPENDENCE (*Sry*-dependent: both direct cell-autonomous effects or indirect testosterone-mediated effects) and SEX CHROMOSOME COMPLEMENT (effects of X and Y-linked genes other than *Sry*) constitute the two factors designated in this thesis.

Several physiological and behavioural phenotypes have now been investigated using the FCG model (Arnold & Chen, 2009). Many phenotypes that have been investigated were shown to be under the influence of *Sry* (i.e. were shown to be *Sry*-dependent effects), thus implying a central role of this pathway as a mediator of sexual differentiation. In terms of physiology, these phenotypes included; plasma testosterone concentration, whereby gonadally male mice had higher levels than gonadally female mice, the number of tyrosine hydroxylase (TH) neurons in the AVPV (gonadally female mice had more neurons than gonadally male mice), the number of motor neurons in the spinal nucleus of the bulbocavernosus (gonadally male mice had more neurons than gonadally female mice), the thickness of the cerebral cortex (gonadally male mice had thicker cortex than gonadally female mice), and the number of progesterone immunoreactive (PR-ir) neurons in the AVPV,

mPOA, and VMN (gonadally male mice had higher PR expression than gonadally female mice) (DeVries, et al., 2002; Gatewood et al., 2006; Markham et al., 2003; Wagner et al., 2004). In terms of behaviour, a *Sry*-dependent effect (sex effect) has been reported for: male copulatory behaviour (gonadally male mice exhibit more than gonadally female mice), social exploration (gonadally male mice explored more than gonadally female mice) chemo-investigatory behaviour (gonadally male mice spent more time investigating a female-soiled bedding than gonadally female mice), parental behaviour (gonadally female mice displayed greater parental behaviour than gonadally male mice), and aggression (gonadally female mice displayed the least aggressive behaviour) (DeVries, et al., 2002; Gatewood, et al., 2006). With regards to parental and aggressive behaviour, it should be noted, that an interaction between *Sry* presence and sex chromosome complement was present, so that XX mice performed in the most effective parental manner and were the least aggressive compared to the rest of the genotypes.

Sex-linked genes other than *Sry* may influence a variety of other phenotypes. For example, using the FCG model the number of vasopressin fibers in the lateral septum, was shown to be sensitive to the effect of sex chromosome complement, as XY carriers had more fibers than XX carriers irrespectively of their hormonal status (DeVries, et al., 2002). Nevertheless, gonadal hormones could also be implicated in this phenotype, as a sex effect, separate from the sex chromosome complement, has also been reported (insofar as gonadally male mice had more fibers than gonadally female mice) (Gatewood, et al., 2006). Similarly, XY carriers have been shown to have higher number of TH-ir neurons in diencephalon and mesencephalon, when taken at embryonic day 14.5 (Carruth, Reisert, & Arnold, 2002); in adult life, however, gonadally female mice, irrespectively of sex chromosome complement, have more TH-ir neurons in the diencephalon (AVPV) than gonadally male mice (DeVries, et al., 2002). Other sex chromosome complement related effects include prodynorphin (Pdyn) expression in the striatum (XX mice had higher expression that XY mice), nociception (XX mice were more sensitive to pain than XY mice), social interaction style (XY mice interacted more with an intruder than XX mice), and habitual responding for food and alcohol reinforcers (for the former, XX mice showed faster habit formation than XY mice, whereas for the latter, the opposite pattern was true) (Barker, Torregrossa, Arnold, & Taylor, 2010; Chen, Grisham, & Arnold, 2009; Gioiosa et al., 2008; McPhie-Lalmansingh, Tejada, Weaver, & Rissman, 2008; Quinn,

et al., 2007). Finally, a recent study reported an interaction between sex chromosome complement and *Sry* in juvenile play behaviour (Cox & Rissman, 2011). Again, these results with the FCG model emphasise the potential complexity of biological mechanisms mediating sex differences in brain and behaviour.

1.3.7 Sex chromosome complement mechanisms underlying sexually dimorphic phenotypes

'SEX CHROMOSOME COMPLEMENT' effects as revealed by models such as the FCG, are as noted earlier distinct from *Sry* effects and could potentially have an effect on sexual differentiation of the brain and sexually dimorphic behaviours via at least three mechanisms: X-linked gene dosage, X-linked genomic imprinting, and Y-linked genes other than *Sry* (Davies & Wilkinson, 2006). The first mechanism, X-linked gene dosage, arises because of two main phenomena: a) males possess one X chromosome, whilst females possess two b) in females there are numerous genes that escape X-inactivation and are located outside PAR, and are thus expressed more highly in females than in males (although not necessarily twice as highly). Briefly, X-inactivation is a dosage compensatory mechanism, which silences one of the X chromosomes in females (Kalantry, 2011). This mechanism has evolved as a by-product of the divergence of sex chromosomes (i.e. sex chromosomes are thought to have evolved from a pair of autosomes around 300 million years ago and diverged, when Y acquired a sex-specific, male determining gene (Vallender & Lahn, 2004)); due to the lack of recombination between X and Y chromosomes, Y-linked genes have degenerated, which has led to silencing of homologous X-linked genes in females, in order to achieve genetic balance between the sexes. In mice, X-inactivation is biased towards the Xp (paternal copy of the X), whereas in humans this mechanism is random (Berletch, Yang, Xu, Carel, & Disteche, 2011). X-inactivation, however, is not complete, and there are genes that escape X-inactivation (notably in PAR). In humans, ~20% of X-linked genes (mostly in the short arm of the X) are thought to escape X-inactivation (Carrel & Willard, 2005), whilst in mice only ~3% of X-linked genes are thought to escape the process (Yang, Babak, Shendure, & Disteche, 2010). In addition, hemizygous males would be more exposed to the phenotypic effects of a hypothetical mutated X-linked gene than females would be,

since females would possess a non-mutated 'compensatory' allele. The imbalance in gene expression between male and female brain as a consequence of X-linked gene dosage may be somewhat ameliorated by the fact that many X-linked genes escaping inactivation have Y-linked homologues. However, often the X-Y pairs are not equal, as X-linked genes are still expressed at a higher level than their Y-linked counterpart (Xu, Burgoyne, & Arnold, 2002). Evidence for an important role of X-linked genes in brain and behavioural phenotypes comes from studies on mental retardation (Hilger Ropers & Hamel, 2005) and schizophrenia (Milunsky, Huang, Wyandt, & Milunsky, 1999). Furthermore, subjects with X-monosomy (Turner's syndrome/TS; 46, XO) or with X-polysomy (such as 47, XXY or 48, XXXY) demonstrate many neuropsychological deficits compared to healthy females (46, XX), and are more prone to developing various neuropsychiatric disorders (Skuse, 2005; Visootsak & Graham Jr, 2006). Animal research has further suggested an important role for X-linked gene dosage, as X-monosomic mice (39,XO) were found to be impaired in similar cognitive domains as patients with TS, compared to their 40,XX littermate controls (Isles, Davies, Burrmann, Burgoyne, & Wilkinson, 2004; P. M. Lynn & Davies, 2007).

In a second mechanism possibly contributing to SEX CHROMOSOME COMPLEMENT effects, X-linked genomically-imprinted genes (genes expressed monoallelically in a parent-of–origin manner (Isles, Davies, & Wilkinson, 2006)) could exhibit a female bias in expression if they are expressed from the paternal X chromosome (since only females inherit an X chromosome from their father) or a male bias in expression, if they are expressed from the maternal X chromosome and the gene in question is subject to X-inactivation (Davies & Wilkinson, 2006). Imprinted genes have recently received a lot of attention and research suggests that they could play an important role in neurodevelopment, cognitive function (Wilkinson, Davies, & Isles, 2007) and vulnerability to neuropsychiatric disorders (Kopsida, Mikaelsson, & Davies, 2011). The main body of evidence on X-linked imprinting stems from studies in TS. In a seminal study, Skuse and colleagues (1997) showed that the neuropsychological profile of patients with TS differs depending on whether they possess a single X chromosome of maternal or paternal origin. Briefly, TS subjects with the karyotype 45,X^pO (i.e. possessing a paternally-inherited copy of the X chromosome) were less impaired in a variety of behavioural tasks (social cognitive skills, verbal IQ and behavioural inhibition) than TS subjects with the

karyotype 45, $X^{m}O$ (i.e. possessing a maternally-inherited X chromosome). Further to that, the latter group was more susceptible to developing autism, a disorder of impaired social cognition. Additional studies on individuals with TS have also indicated distinct cognitive phenotypes in terms of visuospatial and verbal memory ($X^{m}O$ subjects were better in visuospatial tasks and worse in verbal recall compared to $X^{p}O$ subjects; (D. V. M. Bishop et al., 2000)), as well as differences in brain structure (Cutter et al., 2005; Kesler et al., 2003). Work with the 39,XO mouse model has recapitulated some of the above findings in humans in that 39,$X^{m}O$ mice were found to be less behaviourally flexible, when tested in a serial reversal learning paradigm (a task analogous to that applied by Skuse and his team), than 39,$X^{p}O$ mice. Subsequent analysis identified *Xlr3b*, a maternally X-linked imprinted gene, which is subject to X-inactivation, as a potential candidate gene contributing to the development of this specific phenotype (Davies et al., 2005).The closest human orthologue of *Xlr3b, FAM9B*, is deleted in some cases of autism (Thomas et al., 1999) and schizophrenia (Milunsky, et al., 1999). Although *FAM9B* is unlikely to contribute to brain development directly (as its expression is testis-specific ((Martinez-Garay et al., 2002)), there is some evidence from a recent study that it might be associated with serum testosterone levels (Ohlsson et al., 2011). Hence, *FAM9B* could theoretically influence brain function by indirect, hormonally-mediated mechanisms.

The third pathway, which could lead to sexual differentiation, is via Y-linked genes. Genes located in the NRY region of the Y chromosome pass unchanged from father to son and could affect not only testes development and spermatogenesis, but also brain development and behaviour (Kopsida, Stergiakouli, Lynn, Wilkinson, & Davies, 2009). The Y-linked gene which has attracted most interest to date is *Sry* (sex specific region of Y) and this has been discussed above. Other brain-expressed Y-linked genes of potential interest include *Dby*, *Ube1y, Smcy*, *Eif2s3y*, *Uty*, and *Usp9y* (Xu, et al., 2002) (Table 1.3.7i). Most of those genes have X homologues, but research suggests that there are differences in expression patterns between the X and Y linked alleles. *Uty*, for example, has a distinct expression pattern from *Utx* in paraventricular nucleus of the hypothalamus (higher expression than *Utx*) and in amygdala (lower expression than *Utx*) (Xu, Deng, Watkins, & Disteche, 2008). With regards to specific neuropsychiatric disorders, two candidate Y-linked genes should be addressed. The first is *PCDH11Y* and its X chromosome homologue, *PCDH11X*, which encode members of the protocadherin superfamily and are differentially

expressed in the brain (Blanco, Sargent, Boucher, Mitchell, & Affara, 2000). Recent genomewide association studies (GWAS) have implicated *PCDH11X* in late onset AD (Carrasquillo et al., 2009). As females are more vulnerable to developing AD relative to males (Gao, et al., 1998), it is possible that higher *PCDH11X* expression may confer risk of developing the disorder in females, or that *PCDH11Y* expression may confer some degree of neuroprotection to males. Caution though should be exercised, as another recent study failed to replicate this association (including analysis not only in *PCDH11X*, but also in *PCDH11Y* SNPs/single nucleotide polymorphisms) (Miar et al., 2011). The second gene in question is *NLGN4Y* and its X-linked homologue, *NLGN4X*. These genes encode cell adhesion proteins that bind to β-neurexin to form functional synapses and have been implicated in the pathogenesis of autism (Yan et al., 2008). Specifically, studies have shown that mutations on *NLGN4Y* and *NLGN4X* could cause autism and mental retardation in some cases of autism (Jamain et al., 2003; Laumonnier et al., 2004; Lawson-Yuen, Saldivar, Sommer, & Picker, 2008; Yan, et al., 2008). It is unlikely however, that mutations within these genes can account for the majority of cases (Gauthier et al., 2005; Talebizadeh et al., 2006; Ylisaukko-oja et al., 2005).

Lastly, it is of importance to note that interactions between X- and Y-linked genes have been noted, constituting a potential intriguing mechanism towards sexual differentiation. For example, Sry has been shown to regulate *Maoa*, as well as the androgen receptor gene (*AR*), which are both X-linked genes (Tao et al., 2012; Wu, et al., 2009; Yuan, Lu, & Balk, 2001). Furthermore, there is evidence that *Sly*, a multi-copied Y-linked gene, involved in spermatogenesis, and specifically in postmeiotic sex chromatin repression (PSCR) (the phenomenon by which sex chromosomes remain, to an extent, transcriptionally inactive after meiosis) acts by supressing the transcription of spermiogenic genes on the Y, as well as X, chromosome (Cocquet et al., 2009; Ellis, Bacon, & Affara, 2011).

Table 1.3.7i: List of NRY genes potentially important for sexual differentiation (data taken from Xu, et al, 2002, and http://mouse.brain-map.org).

Gene	Expression pattern	Function
Dby	Testis, muscle, liver, heart, kidney, and brain (low expression in cortex, and hippocampus).	Involved in initiation of translation.
Ube1y	Testis and brain (expressed throughout, including cortex, hippocampus, striatum, cerebellum, midbrain, and diencephalon).	Involved in protein degradation.
Smcy	Testis, muscle, liver, heart, kidney, and brain (olfactory areas and medulla).	Encodes peptides corresponding to epitopes of the male-specific antigen, H-Y (Agulnik, Mitchell, Lerner, Woods, & Bishop, 1994).
Eif2s3y	Testis, muscle, liver, heart, kidney, and brain (expressed throughout, including cortex, hippocampus, striatum, cerebellum, midbrain, and diencephalon).	Involved in initiation of translation.
Uty	Testis, muscle, liver, heart, kidney, and brain (mainly expressed in cortex, olfactory areas, thalamus and midbrain).	Involved in protein-protein interactions.
Usp9y	Testis, and developing brain (but not adult).	Involved in protein degradation. Furthermore, Usp9x/y could mediate neuronal development.

1.3.8 Limitations of the FCG mouse model

One of the most important advantages of employing mouse models is that they allow for experimental control that is not possible with human subjects. Mice can be easily bred and maintained, as well as genetically-manipulated. In the field of neuroscience, transgenic and knockdown mouse models have been widely used, as they can provide insight on the role of specific gene products and their downstream pathways. To date,

genetically-manipulated mouse models have been developed for various neuropsychiatric disorders, such as depression (Hoyle et al., 2011), Alzheimer's disease (AD) (Marchetti & Marie, 2011), Parkinson's disease (PD) (Schmidt et al., 2011), and childhood-onset disorders (Robertson & Feng, 2011). Furthermore, mice, being mammals, bear many similarities with humans, in terms of their genetic composition (Tecott, 2003) and gross neurobiology (Arguello & Gogos, 2006; Cryan & Holmes, 2005; Han & Gu, 2006), which makes data extrapolation between the species possible with caution.

In the field of sex differences, many genetically-manipulated models have been developed and characterised. These models include mice with manipulations of steroid hormonal receptors (e.g. the 'Tfm' model), of gene products known to be fundamental in sexual differentiation (e.g. the Sf1 transgenic model), or of the SEX CHROMOSOME COMPLEMENT (39,XO model) (Arnold, 2009a; P. M. Lynn & Davies, 2007; Zuloaga, Puts, Jordan, & Breedlove, 2008). The FCG constitutes one such model, and its utility for dissociating between the effects of *Sry* and other sex-linked genes on neural and behavioural processes has been discussed above. However, the FCG model also has some limitations, which should be taken into account in the design and interpretation of experiments.

One potential issue is the possibility that XX and XY mice of the same gonadal status (XX and XY-; XX*Sry* and XY-*Sry*) might differ in the morphology and function of their gonads. It has been suggested, for example, that XY- female mice are less fertile than normal XX female mice (Lovell-Badge & Robertson, 1990). Analysis of gonadally mediated phenotypes, though, such as oestrous cycle (P. M. Y. Lynn, 2010), male copulatory behaviour (DeVries, et al., 2002), and testosterone levels (Gatewood, et al., 2006), have not yielded any differences between XX and XY mice of the same gonadal sex, which indicates that they have similar gonadal function. A second set of limitations relates to the potential influences of prenatal hormones, hormonal secretions from other tissues, as well as unique characteristics of the *Sry* transgene (Ngun, et al., 2011). With regard to the latter, behavioural differences have been reported between XY wildtype and XY-*Sry* mice (male copulatory behaviour, social exploration;(DeVries, et al., 2002)), and data from our own group suggest that *Sry* expressed transgenically is expressed higher in the brain than endogenous *Sry* (i.e. *Sry* expressed from its normal location on the Y chromosome) (P. M. Y. Lynn, 2010).

Therefore, extrapolating data from the FCG model to normal wildtype rodents (and thereafter to humans) might not be straightforward.

A third and very crucial point that should be addressed is the lack of specificity in terms of the mechanisms driving the observed effects. As it has already been mentioned, there are three main ways via which sex-linked genes could act (X-linked dosage, X-linked imprinting, and Y-linked genes); thus any FCG phenotype related to sex chromosome complement could be caused by one or several of these three mechanisms. Similarly, for *Sry*-dependent phenotypes, it is not possible, using this model in isolation, to dissociate between direct effects that could be attributed to the action of the gene *per se* (irrespectively of hormonal secretions) and indirect effects that are mediated by the actions of testosterone. Some of these problems have previously been addressed through gonadectomising adult mice and administering equal levels of gonadal hormones (Arnold & Chen, 2009; Gatewood, et al., 2006; Gioiosa, et al., 2008). In this way, the post-natal hormonal environment should be similar among the male and female genotypes. However, this strategy introduces additional confounds, in that the surgical procedure might interact with sex to influence subsequent performance in behavioural tasks e.g. via differential anaesthetic effects on the brain, or via differential reactivity to stress and recovery. Additionally, by equalising the adult hormonal environment, no information could be provided with regards to activational effects of gonadal hormones. Finally, gonadectomising adult mice will not eliminate the important organisational effects of gonadal hormones which predominantly occur *in utero*.

When working with mouse models, including FCG, there are a number of general issues that need to be considered, such as the potential confounds of using different strains, especially as behavioural differences among strains have been reported widely (Van Meer & Raber, 2005). According to a meta-analysis of commonly used inbred strains, significant differences exist for behavioural paradigms, such as locomotor activity, EPM, and ethanol preference (Whalsten, Backmanov, Finn, & Crabbe, 2006). One good example is the C57BL/6J strain, which has been shown to be generally more active, and to display less anxiogenic behaviour compared to other inbred stains (Tarantino, Gould, Druhan, & Bucan, 2000; Whalsten, et al., 2006). These inter-strain differences could also be of relevance to previous studies conducted using the FCG model, as more than one background strain has been used; some studies have used the C57BL/6J strain to generate FCG mice

(Gatewood, et al., 2006; McPhie-Lalmansingh, et al., 2008), whereas in other cases transgenic mice were generated on a MF1 background (DeVries, et al., 2002; Quinn, et al., 2007). Caution thus should be exercised, when interpreting results, especially since in the case of FCG mice C57BL/6J is an inbred strain, whereas MF1 is an outbred stock. Outbred strains, contrary to inbred, have high degrees of genetic variability and could carry mutations, which could affect experimental results (by either accounting for the results or by interacting with *Sry* and/or other sex-linked genes). Notwithstanding, outbred strains could be useful, as they may mimic in a more reliable way human genetic variability (Chia, Achilli, Festing, & Fisher, 2005).

Other caveats associated with the use of mouse models include discrepancies in sex chromosomal functions between mice and humans, notably in terms of X-inactivation profiles (see section 1.3.1), as well as genetic composition in the Y chromosome and PARs (fewer genes in mice than in humans; (Davies, 2011; Flaquer, Rappold, Wienker, & Fischer, 2008)). Finally, it should be acknowledged that some aspects of normal human function and disorders, such as language/language impairments and psychosis, remain essentially outside the boundaries of what can be modelled in rodents.

1.4 Specific aims of the thesis

The main aim of the thesis was to further investigate the role of *Sry* and other sex-linked genes in brain and behaviour. The experimental work used two rodent models. In Chapters III, IV and V, the above discussed FCG cross was employed to identify specific evidence for *Sry*-dependent and/or sex chromosome complement on aspects of emotional behaviour. Additionally, the data obtained using the FCG mouse model were further assessed in terms of any SRY-DEPENDENCE effects being due primarily to direct cell-autonomous effects, or more closely related to the more established mechanisms involving Sry effects on testosterone secretion; this was achieved by correlating behavioural measures with brain-expressed *Sry* and serum testosterone levels. In the final experimental chapter, Chapter VI, new 'gene knockdown' methods were developed to enable a direct manipulation of *Sry* in the adult rat brain (specifically in the ventral tegmental area) in order to be able to test the effects of Sry in this brain area on emotional behaviours that had been shown to be sensitive to effects in the FCG model in hormonally-intact animals. In Chapter VII, the General Discussion, the data are discussed in terms of the limitations of both

models used in the thesis, the pattern of data suggesting, in particular, Sry-dependent effects on emotional functioning and other behavioural findings which could not be related to Sry-dependent effects but were instead related to sex chromosome complement. Finally, a synthesis of the data is attempted in terms of the physiological relevance of the data to sex differences in brain and behaviour and its possible clinical relevance.

Chapter II

General Materials and Methods

This chapter includes general procedures that were performed routinely during the course of the thesis, and descriptions of the behavioural apparatus used. All procedures involving the use of live animals were carried out in accordance with the requirements of the U.K Animals (Scientific Procedure) Act (1986) and in line with the Home Office Project Licences granted to Dr. William Davies (PPL 30/2601) and Prof. Lawrence Wilkinson (PPL 80/1937). Experimental work was performed under the Home Office Personal Licence granted to Eleni Kopsida (PIL 30/8333).

2.1 Subjects and animal husbandry

2.1.1 Four core genotype mice

The 'four core genotype' (FCG) mice used were originally generated by our collaborator Dr. Paul Burgoyne in the MRC National Institute for Mental Research (NIMR), Mill Hill, London on an MF1 strain background, using the crosses outlined in the General Introduction (Lovell-Badge & Robertson, 1990; Mahadevaiah, et al., 1998). In an attempt to reduce variability between animals, the original FCG cross possessed the so-called 'uniform X' chromosome (details in Figure 2.1.1a). For the experiments conducted as part of this thesis, this 'uniform X' line was established at the Behavioural Neuroscience Laboratory of the School of Psychology, at Cardiff University, from animals that were in turn originally transported from the Babraham Institute, in Cambridge in 2006. Briefly, XX females (possessing the 'uniform X' chromosome) were paired with XY-*Sry* males, in order to generate the four genotypes (XX, XY-, XY-*Sry*, and XX*Sry*, see Figure 2.1.1a).

Prior to weaning, pups were housed with the mothers in environmentally enriched cages (i.e. with cardboard tubes, shred-mats, tissue paper) in a temperature and humidity controlled holding room (21 ± 2°C and 55 ± 10% respectively), with a 12-hour light-dark cycle (lights on at 7:00 hours/lights off at 19:00 hours). Following weaning, mice with the same gonadal genotype were housed together (2-5 per cage; same environment as above), but each cage sometimes included mice of more than

one genotype (i.e. for males XY-*Sry*, and XX*Sry*; for females XX, and XY-). Wildtype XY mice were housed separately (see section 2.1.2 below). Standard mouse chow and water were available *ad libitum (ad lib)* unless stated otherwise. Homecages were cleaned and changed once per week, on the same day, and at approximately same time of the day, in order to minimise potential disruption to the behavioural testing. All experimental animals were regularly weighed and monitored for signs of ill health. Any mice which appeared unwell were immediately assessed by the NACWO animal technician and the vet. Sentinel mice housed in the same environment with the experimental animals were regularly assayed for pathogens at Harlan, U.K.

Figure 2.1.1a

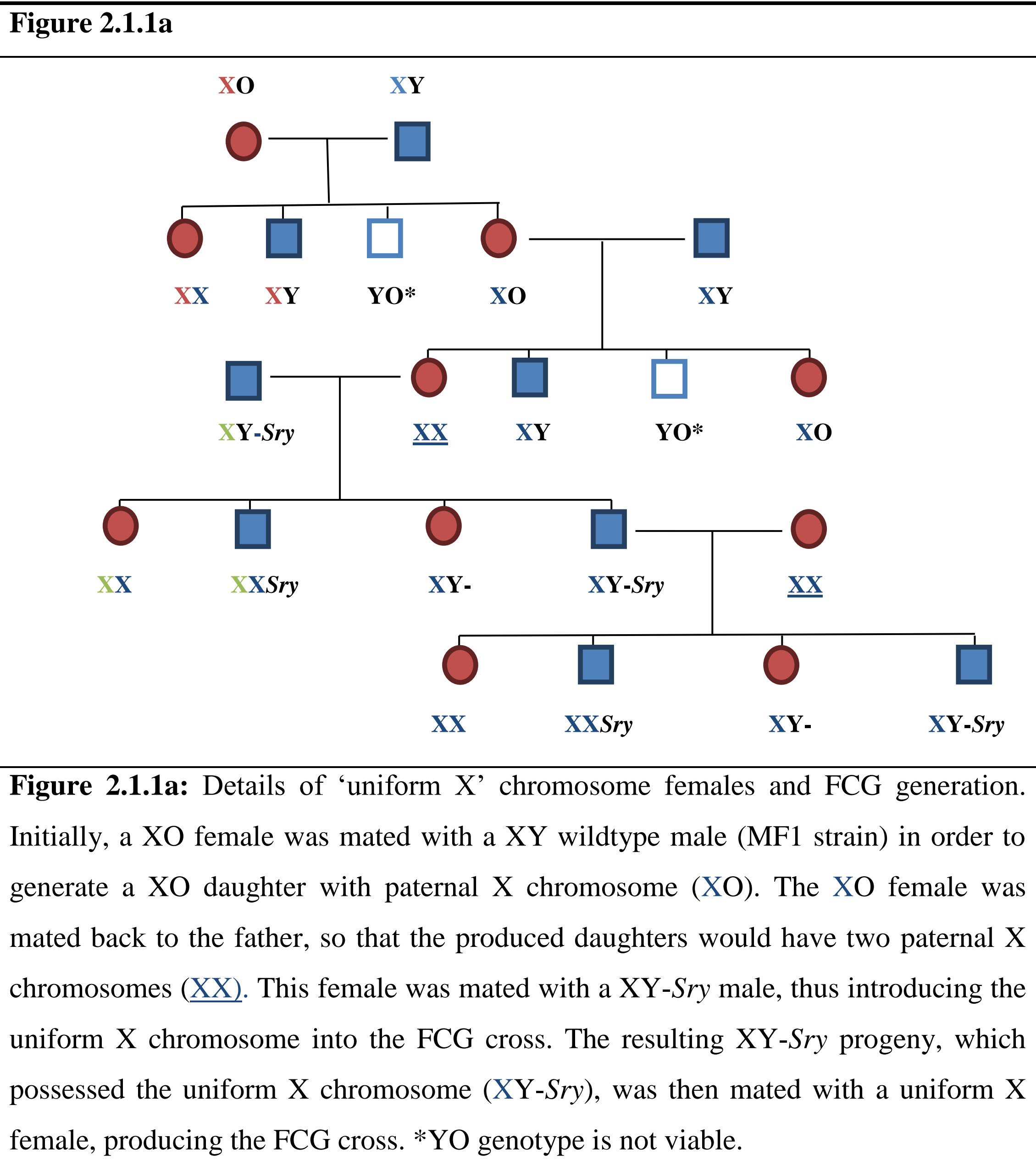


Figure 2.1.1a: Details of 'uniform X' chromosome females and FCG generation. Initially, a XO female was mated with a XY wildtype male (MF1 strain) in order to generate a XO daughter with paternal X chromosome (XO). The XO female was mated back to the father, so that the produced daughters would have two paternal X chromosomes (XX). This female was mated with a XY-*Sry* male, thus introducing the uniform X chromosome into the FCG cross. The resulting XY-*Sry* progeny, which possessed the uniform X chromosome (XY-*Sry*), was then mated with a uniform X female, producing the FCG cross. *YO genotype is not viable.

2.1.2. XY wildtype mice

In addition to the FCG model animals, wildtype 40, XY male mice were occasionally generated at the animal laboratory, at Cardiff University. Also, for the specific experiment detailed in Chapter V, 64 MF1 40, XY mice were transported from Harlan (Harlan, ORLAC, Bicester, U.K.) to Cardiff. All wildtype male mice were housed (2-5 per cage) in environmentally enriched cages, and handled following the same protocol as for the FCG mice.

2.1.3 Lister-Hooded male rats

For the purposes of the last experimental chapter, Chapter VI, adult male Lister-hooded rats, weighing around 250-275gr., were transported from Harlan (Harlan, ORLAC, Bicester, U.K.). Animals were housed in pairs, in environmentally enriched cages (i.e. cardboard tubes, chew-sticks) and had *ad lib* access to food (standard rodent chow) and water. A two weeks acclimatization period preceded any experimental procedure. As with the mice, any health issue was reported to the NACWO animal technician. Home cages were cleaned and changed once per week, as stated in section 2.1.1.above. Rats were only used in a single experiment in the thesis and so the majority of the general methods detailed here relate to the mouse work that follows in experimental Chapters III, IV, and V. Relevant details of methodological approaches for the work using rats can be found in Chapter VI.

2.2 <u>Habituation to handling, test environments and ancillary procedures</u>

2.2.1 Handling and body weight measurement

All animals were allowed to acclimatize to the general housing environment to be used for the duration of the experiments for at least two weeks, before being handled by the experimenter for a further two weeks (approximately 1 minute per animal per day). During this procedure body weights were measured regularly and recorded at the same time each day (around 17:00, following behavioural testing; and if animals were on a water restriction schedule [see immediately below], two hours after access to water).

2.2.2 Protocol for water restriction schedule

Prior to behavioural testing where liquid reinforcer was used as reward, subjects were placed on a water restriction schedule used routinely in the laboratory in order to motivate performance in the behavioural tasks. According to this protocol, animals initially had 4 hours access to water for 4 days, and 2 hours access thereafter until the end of the experimental procedure. During the period of experimentation the 2 hours access to water was always at the end of behavioural testing. For the initial two week period of acclimatization to the restricted water schedule the water bottles were weighed at the start and end of water access, to ensure that animals were drinking sufficient amounts of water. Additionally, every two weeks animals were allowed free access to water for 24 hours. Animals showing signs of dehydration or having their body weight reduced >20% were immediately given *ad libitum* access to water, until their body weight was stabilized to *ad libitum* weight levels. Standard laboratory chow was available *ad libitum* throughout the duration of the water restriction schedule.

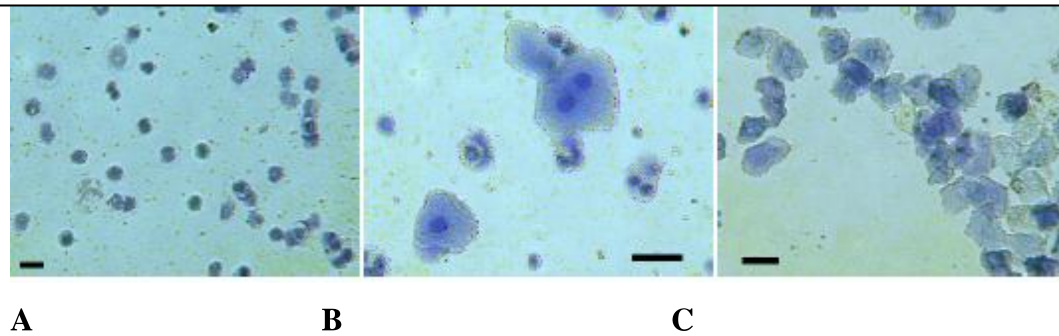
2.2.3 Reinforcer habituation/preference test

Once the body weight of animals had stabilized following instigation of the water restriction schedule, subjects were habituated to the reinforcer used in the appetitively motivated behavioural tasks (10% condensed milk solution, Nestle Ltd, U.K.). The test, which also provided a measure of any inherent pre-existing differential preference for the reinforcer, occurred in five holding cages (285 x 130 x 120mm, one mouse per cage) over five consecutive days, for a 10 minutes session each day. The cages did not contain sawdust, were empty, and had only two containers (diameters 25mm), with adequate volume (approximately 6ml) of liquid, affixed to the bottom of the cage. Containers were weighed prior and after daily testing, to ascertain liquid consumption. During the first testing day, which served as a habituation session to the apparatus and a measure of general water consumption, both containers were filled with water. For the following four days, one container held water and one container held the reinforcer 10% condensed milk solution. Between sessions, the position of containers was switched to avoid positional response bias. Reinforcer preference was defined as the amount of liquid consumption, as a percentage of the total amount of liquid (water + reinforcer) consumed over the four testing days.

2.2.4 Vaginal smearing

To determine the oestrous status of female mice, vaginal smearing was performed with a cotton wool swab. The procedure was very quick and did not appear to cause the mice any discomfort. Cells were subsequently stained in 0.05% cresyl violet solution for approximately seven minutes. Smearing was performed at the end of each testing day. Stage of oestrous (dioestrous, proestrous, oestrous) was determined based on the morphology of cells (Figure 2.2.4a).

Figure 2.2.4a



A **B** **C**

Figure 2.2.4a: A. Dioestrous stage, characterized by small leucocytes. **B.** Proestrous stage, characterized by nucleated cells. **C.** Oestrous stage, characterized by large cornified cells. Bar = 10µm. Photos courtesy of Dr. William Davies.

2.2.5 Behavioural testing environment

All behavioural testing was carried out in sealed and air-conditioned testing rooms. Rooms were lit by fluorescent lights, which could be adjusted to desirable levels. Although temperature and humidity levels were not strictly controlled, these parameters were generally maintained around 21 ± 2°C and 55 ± 10% respectively. Rooms were thoroughly cleaned once per week.

2.2.6 Additional experimental control measures

Throughout the behavioural testing, additional control measures were employed in order to minimize experimental noise and to ensure greater consistency of the data. Behavioural testing always occurred during the light phase of the light-dark cycle of the holding room (between 07:00 and 19:00), and water access (in cases of water deprivation schedule) was always administered immediately upon the completion of testing. Furthermore, to minimize order effects, animals were tested in a pseudorandom order. Potential confounds associated with cage/litters, were addressed by having the experimental subjects drawn from a large number of cages/litters as available.

2.3 Behavioural apparatus

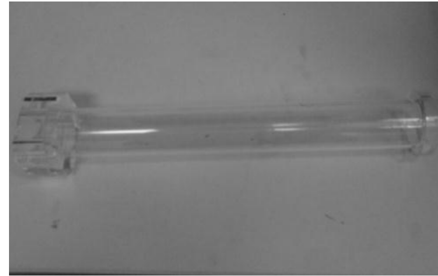
2.3.1 Mouse behavioural apparatus

2.3.1.1 Phenotyper cages

A continuous 24hr period of behaviour was measured using four phenotyper cages (Noldus, U.K.). Each cage (300 x 300 x 350mm, length, width, height), made of clear Perspex, contained the following:

- a running wheel (light blue acrylic, 150mm diameter).
- a shelter (infrared translucent Perspex, 100 x 100 x 50mm, length x width x height).
- a lickometer.
- a feeder.

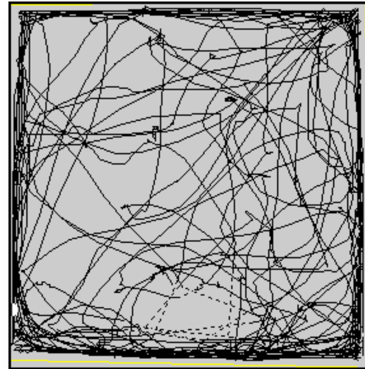
The bottom of the cage was covered with black paper to facilitate recording. Both running wheel and lickometer were connected to an electronic box through a cable, for movement detection. The top unit had a camera with infrared arrays to record performance.

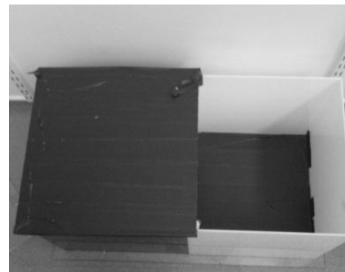
2.3.1.2 Social dominance tube test	
A tube (300mm length, 30.5mm diameter), made of clear Perspex, was used to assess social dominance. Mice were placed facing the two ends of the tube and were released simultaneously. A win score was given to the mouse that succeeded in ousting its opponent from the tube. Performance was scored by the experimenter.	

2.3.1.3 Locomotor activity boxes	
Locomotor activity (LMA) was tested in 12 clear Perspex boxes (each 210 x 360 x 200mm, width x length x height), with one animal in each box. Two infra-red beams crossed each cage 30mm from each end and 10mm from the floor of the chamber. Beam breaks data were recorded by a computer with custom written BBC Basic V6 programmes (Cambridge Cognition Ltd., Cambridge, U.K.).	

2.3.1.4 Elevated Plus Maze (EPM)	
The EPM consisted of two exposed open arms (175 x 78mm, length x height) and two enclosed arms (190 x 80 x 150mm, length x width x height) with an open roof. Equivalent arms were arranged opposite one another. The plus maze was constructed of dulled black Perspex (7mm thick), was elevated 300mm above the floor and dimly illuminated(~10lux). Subjects were tracked using the Ethovision tracking system (Noldus, U.K.).	

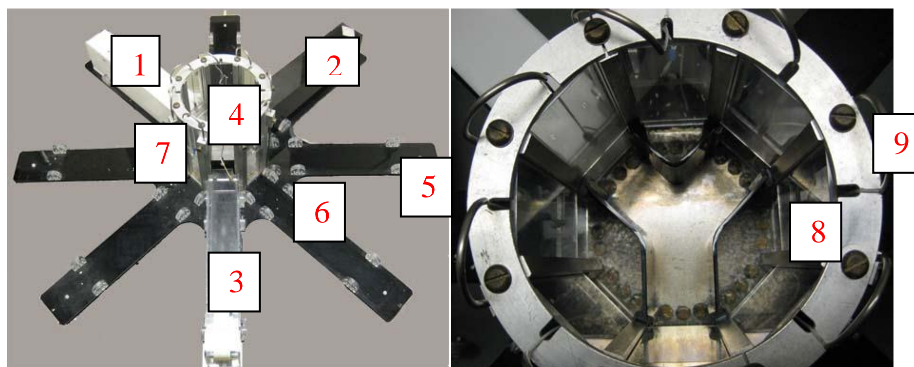
2.3.1.5 Elevated Zero Maze	
The Zero Maze (600mm in diameter) consisted of two open quadrants and two enclosed quadrants (220mm height) with an open roof. The maze was elevated 500mm above the floor and dimly illuminated (~10lux).Performance was recorded using the Ethovision tracking system (Noldus, U.K.).	

2.3.1.6 Open Field arena	
The open field apparatus consisted of a square-shaped arena (750 x 750mm, length x width), constructed of white plastic. For Ethovision tracking purposes, black plastic was placed under the white transparent area. The arena was dimly illuminated (~10lux).Animals were tracked using the Ethovision tracking system (Noldus, U.K.).	

2.3.1.7 Light-Dark Box	
The light-dark box (300 x 600 x 300mm, width x length x height) was made of Perspex and separated into two equally sized compartments. The dark compartment (black Perspex) had a black roof, whereas the light compartment (white Perspex) was open and brightly lit by a 60W lamp (~150lux). Free access to both compartments was allowed through a partition door (70 x 70mm, width x length). Data were recorded using the Ethovision tracking system (Noldus, U.K.).	

2.3.1.8 Apparatus for the visual discrimination learning task

An 8-arm radial maze (Tech^{nix},Babraham Institute, U.K.), converted for use as a Y-maze, was used for the visual discrimination learning task. The apparatus was comprised of two Perspex goal arms (one black, one white), and one transparent Perspex start arm, of equal dimensions (345 x 51 x 81mm, length x width x height). All arms were detachable and had sensors attached to them in three different positions; at 20, 70, and 280mm from the centre of the maze. These sensors served to record the activity of the animal as it moved in the maze and were connected to motors (Amerang Ltd., U.K.), which controlled the Perspex guillotine doors at the centre of the maze. Each goal arm had, at 320mm from the centre of the maze, a shallow well, where the reinforcer was placed, without being visible from the central area. The maze was operated and performance was recorded using an Acorn computer (ARACHNID programme, Cambridge Cognition Ltd., U.K.) with custom written software.

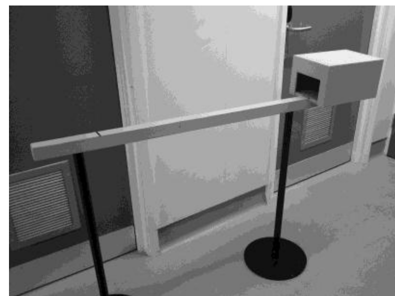


1 white goal arm, **2** black goal arm, **3** start arm, **4** central area, **5** infra-red sensor at 280mm, **6** infra-red sensor at 70mm, **7** infra-red sensor at 20mm, **8** "choice space", **9** transparent guillotine door.

2.3.2 Rat behavioural apparatus

2.3.2.1 Locomotor activity boxes

Locomotor activity (LMA) was tested in eight clear plastic boxes, similar to the ones described in section 2.3.1.1. Locomotor activity chambers (500 x 360 x 200mm, length x width x height) had infra-red beams crossing them, in order to record performance of the animal. Beam breaks were recorded by an Acorn 1989 computer (ARACHNID programme, Cambridge Cognition Ltd., U.K.).

2.3.2.2 Balance beam	
Motor function was assessed with a wooden, narrow balance beam (1050 x 40 x 30mm, length x width x depth). The beam was elevated 80cm from the ground by metal supports at either end. Beneath the beam, foam pudding was placed to prevent injury of the animals in case of fall. At the end of the beam, there was a 300 x 210 x 160mm (length x width x height) box where the rat could enter (partition: 140 x 80 mm; height x width), until the experimenter placed him back in his home cage. Saw dust taken from the animal's cage was placed inside the box, to make it a familiar and less aversive area.	

2.3.2.3 Elevated Plus Maze (EPM)

The rat EPM utilized in the thesis was very similar to the murine EPM (section 2.3.1.4) only bigger. The EPM consisted of four black, wooden arms (500 x100mm, length x width), elevated 500mm from the ground. The two enclosed arms had 300mm high walls and were arranged opposite each other.

2.4 Culling protocol

The protocol described here applies only to mice used as experimental subjects. Details on the protocol used for the experiment conducted in rats can be found in Chapter VI. Briefly, at the end of the experimental procedure, or in case of persistent illness, animals were culled through cervical dislocation. Brain tissue was collected, as well as trunk blood samples.

2.5 Genotyping protocol

2.5.1 DNA extraction

Plucks of hair were taken from the FCG mice after weaning, for DNA extraction. Hair plucking was used, as an alternative to tail biopsy, because of minimal distress effects on the animals (Schmitteckert, Prokop, & Hedrich, 1999). Samples were placed in 1.5ml micro-centrifuge tubes, and 50µl of NaOH was added to each tube. Samples were spun for 1 minute in a centrifuge at 13.000 rpm, in order to collect the hair on the bottom of the tube. The tubes are heated for 10 minutes at $95^{o}C$ to dissolve chromosomal DNA, and subsequently placed in wet ice to chill. Afterwards, the lysates were centrifuged again for 30 seconds. 3µl of the DNA sample were used in a 25µl PCR reaction.

2.5.2 Polymerase chain reaction (PCR) for genotyping

To determine the sex chromosome complement of the FCG mice (whether they possess XX or XY), a PCR reaction was performed with *Ssty* as the targeted Y-linked gene. *Om1a* (myogenin) served as an amplification control. Reaction mixture was produced in accordance with the PCR protocol (Table 2.5.2i.). It was then transferred to a PCR machine (MJ Research, U.K.) and was run using the programme outlined in Table 2.5.2i.

Table 2.5.2i: Details of the PCR protocol used for genotyping

Reaction volume	**Programme-cycle**
15.75μl of nuclease-free H_2O	**1**. 94°C for 15min.
2.5μl of 10x buffer	**2**. 94°C for 45secs.
1μl of of 5mM dNTPs	**3**. 55°C for 45secs.
1μl of 10μM *Ssty* forward primer (5'CTGGAGCTCTACAGTGATGA3')	**4**. 72°C for 45secs.
1μl of 10μM *Ssty* reverse primer (5'CAGTTACCAATCAACACATCAC3')	**5**. Go to Step 2; 40 times.
0.25μl of 10μM *Om1a* forward primer (5'TTACGTCCATCGTGGACAGCAT3')	**6**. 72°C for 5min.
0.25μl of 10μM *Om1a* reverse primer (5'TGGGCTGGGTGTTAGTCTTAT3')	**7**. 4°C forever.
0.25μl of Taq polymerase buffer	**8**. End.
3μl of DNA	
25 μl	

PCR products were visualized on an agarose gel stained with 1.5% ethidium bromide using a UV scanner. Samples with two amplicons possessed a Y chromosome (XY-*Sry* for gonadally males, and XY- for gonadally females), whereas samples with only the *Om1a* gene amplified were carriers of two X chromosomes (XX-*Sry* for gonadally males, and XX for gonadally females) (Figure 2.5.2a).

Figure 2.5.2a

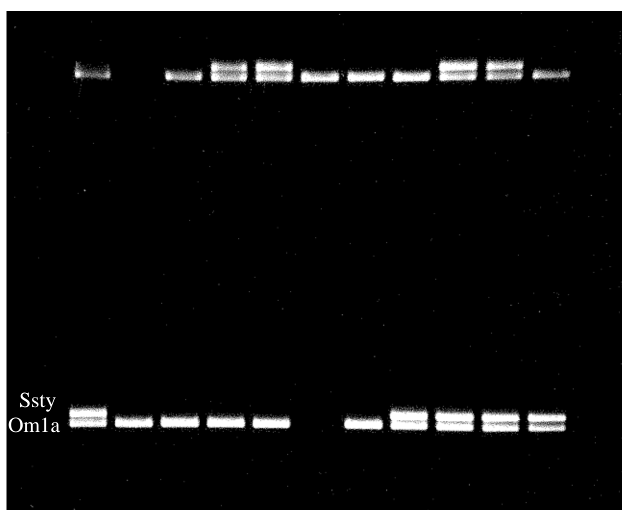


Figure 2.5.2a: Example of agarose gel visualized under UV light. Samples with two bands are karyotypically males. *Ssty* product is heavier (343bp) than *Om1a* (245bp), and therefore travels less along the gel.

2.6 Testosterone blood level analysis; ELISA protocol

Following culling by cervical dislocation, trunk blood was collected and transferred to blood collection tubes (BD Microtainer tubes, gold; BD, U.S.A.). Serum was separated and stored at –20°C in eppendorf tubes. Testosterone levels were assayed by a testosterone ELISA kit (DRG Instruments, GmbH, Germany) according to the protocol provided. In detail, microtitre wells coated with a mouse monoclonal anti-Testosterone antibody were placed in a plate reader. 25μl of each thawed serum sample, standard (solutions with testosterone concentrations at 0, 0.2, 0.5, 1, 2, 6, and 16 ng/ml; included in kit), and control sample (deionised water) were loaded in each well. 200μl of enzyme conjugate was added into each well, and mixed thoroughly. Wells were incubated for an hour at room temperature. The contents of the wells were subsequently shaken out, and the wells rinsed three times with diluted wash solution (400μl per well). Samples were then incubated for 15 minutes, after having 200μl of substrate solution added to each well. The enzymatic reaction was terminated by adding 100μl of stop solution. The optical density (450 ± 10nm) was read with

Sunrise, a microplate calibrated reader (Tecan Group Ltd, Switzerland), running on the programme XFluor4 (Tecan Group Ltd, Switzerland).

The optical densities of all samples were plotted, using SigmaPlot (SYSTAT, U.S.A.), on a standard curve graph, where the X axis was the testosterone concentration and the Y was the absorbance value. The standard curve was modeled using the "Hyperbola 3 decay" model (non-linear regression):$f=y0+(a*b)/(b+x)$; y0, a, and b are constants to be found; f corresponds to the optical density reading of the serum samples. This equation was then used to calculate the sample concentrations of testosterone.

2.7 Brain dissection

In several cases brain tissue was collected upon completion of experiments for use in quantitative gene expression analyses. Dissection was performed with a razor blade (one blade per sample) on a metal plate pre-cooled with dry ice. This protocol facilitated the dissecting procedure, by making the tissue more amenable to cutting and also limited structural and metabolic degradation during dissection. .For the mouse work in Chapters III and V, cerebral tissue from whole half brain was collected (excluding the olfactory bulbs) with a sagittal dissection along the central sinus. Gene expression analysis of brain tissue in the rat experiment (Chapter VI) was restricted to tissue collected from the ventral tegmental area (VTA) of the brain (Figure 2.7a). VTA was dissected from a coronal section (2-3mm) at -5.3mm from bregma (Paxinos & Watson, 1998). All dissected tissue was immediately frozen on dry ice, and subsequently stored in sterile containers at -80°C.

Figure 2.7a

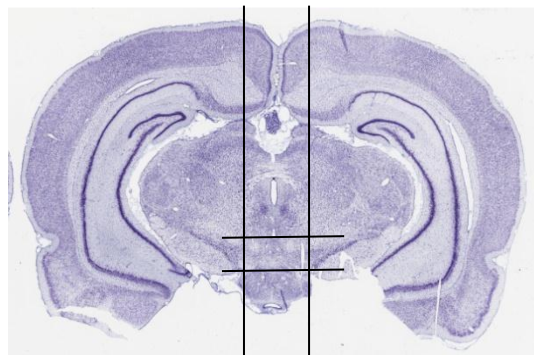


Figure 2.7a: A schematic rat coronal section, from where VTA was dissected (right). The brain image was obtained from http://brainmaps.org.

2.8 Quantitative gene expression methods

2.8.1 RNA extraction

In order to quantify gene expression levels cerebral tissue from extracted brains was transferred to lysing matrix tubes (MB Biomedicals) and 1ml of TRI reagent (Sigma-Aldrich, U. K.) was added to each tube. Samples were homogenized in a ribolyser machine (Hybaid Ltd., U.K.) twice for 15 seconds, at the speed rate of four. Subsequently, 200µl of chloroform was added to each lysing tube, and shaken for 10 seconds. The mixture was centrifuged for 15 minutes at 4000rpm and 4°C. Next, the top clear supernatant was transferred to a new eppendorf, and 500µl of isopropanol was added. Samples were again centrifuged for 10 minutes at 4000rpm and 4°C. The supernatant was then poured off retaining the pellet in the eppendorf. 1ml of 75% ethanol was added, and eppendorfs were vortexed for 10 seconds, and centrifuged for 5 minutes. Afterwards, the supernatant was drained and the tubes were allowed to dry, by heating them on the heating block at 50°C for 30 minutes. 50µl of nuclease-free water was also added to the eppendorfs, while on the heating block, and samples were vortexed really well. Finally, a spectrophotometer (NanoDrop, Thermo Scientific, U.S.A.) was used to determine the quality and concentration of RNA in each sample

(optical density at 260 and 280nm). All samples received DNAase treatment in order to remove residual DNA; 5µl of DNAase buffer (x10) and 1µl of DNAase (Ambion, U.S.A.) were added to each tube. Samples were placed on the heating block, initially for 30 minutes at 37°C, and then for 15 minutes at 80°C.

2.8.2 cDNA synthesis

Synthesis of cDNA was performed using Sprint RT Complete-Random Hexamer tubes (Clontech, U.S.A.). 1µg of DNA-free RNA, made up to a final solution of 20µl with nuclease-free water, was added to the tubes. Samples were then incubated for 75 minutes at 42°C, and for 10 minutes at 80°C, to terminate the reaction. Finally, 180µl of water was added to the 20µl mixture to make up a total 200µl solution.

2.8.3 Quantitative PCR

Gene expression was measured using a Rotorgene 6000 RT-PCR machine (Corbett Research, U.K.) following the protocol outlined in Table 2.8.3i. The qPCRmastermix was prepared using a PCR Setup pipetting robot (CAS-1200, Corbett Life Science, Qiagen, U.S.A.) in order to reduce potential human errors in pipetting and to ensure consistency between qPCR runs. Details of the primer sequences used in Chapters III, V, and VI can be found in Table 2.8.3ii. Samples were run in at least duplicate. Three non-template controls (5µl of water per sample, instead of DNA) were included in each qPCR run.

Table 2.8.3i: General qPCR protocol used throughout the thesis.

Reactions	Programme
1.9µl of H_2O	1. 95°C for 10’
7.5µl of SensiMix SYBR (Quantace)	2. 95°C for 15’’
0.3µl forward primer	3. 55°C for 20’’
0.3µl reverse primer	4. 72°C for 15’’
5µl of diluted cDNA sample	5.Go to step 2; 40 times (55 for *Sry* reaction)
Total: 15µl	6. Ramp from 60°C to 90°C rising 1°C each step (melting curve analysis)
	7. Wait for 90’’ of pre-melting conditioning on first step, and for 5’’ for each step afterwards
	8. End

Table 2.8.3ii: List of primer sets used in the thesis.

Gene	Primer direction	Primer sequence
Hprt	Forward	5’TTGCTCGAGATGTCATGAAGGA3’
	Reverse	5’AATGTAATCCAGCAGGTCAGCAA3’
Gapdh	Forward	5’GAACATCATCCCTGCATCCA3’
	Reverse	5’CCAGTGAGCTTCCCGTTCA3’
Dynein	Forward	5’GGACATTGCTGCCTATATCAAGAAG3’
	Reverse	5’CGTGTGTGACATAGCTGCCAA3’
B-actin	Forward	5’TCTGTGTGGATTGGTGGCTCTA3’
	Reverse	5’CTGCTTGCTGATCCACATCTG3’
Sry(mouse)	Forward	5’TTTCCAGGAGGCACAGAGAT3’
	Reverse	5’GCAGGCTGTAAAATGCCACT3’
Sry(rat)	Forward	5’CCAGTCCTCCAAGAACCAGA3’
	Reverse	5’TAGTGGAACTGGTGCTGCTG3’
Th	Forward	5’TGTGTCCGAGAGCTTCAATG3’
	Reverse	5’GGGCTGTCCAGTACGTCAAT3’
Maoa	Forward	5’ACCAATTAATTCAGCGTCTTCCA3’

	Reverse	5’ATCATGCAGCCACAATAGTCCTT3’
Tph2	Forward	5’CTGCTGTGCCAGAAGATCATCA3’
	Reverse	5’TGCTGCTCTCTGTGGTGTCG3’
Fosl1	Forward	5’AGCCCATCGAAAGAGTAGCA3’
	Reverse	5’GATGACAACGGGTAGCACCT3’
Plp	Forward	5’ACCACCTGCCAGTCTATTGC3’
	Reverse	5’GAAAAGCATTCCATGGGAGA3’
Sst(Somatostatin)	Forward	5’CCCAGACTCCGTCAGTTTCT3’
	Reverse	5’GGCATCGTTCTCTGTCTGGT3’
Gat-1	Forward	5’GCCTGGGTGCTTGTGTATTT3’
	Reverse	5’CCACGGAAGAACAGGATGAT3’

2.8.4 Analysis of qPCR measurments: the 2 $^{-\Delta\Delta ct}$ method

For data analysis, a relative quantification method was applied, as the aim was to compare gene expression among different groups. Hence, the 2 $^{-\Delta\Delta ct}$ method was used, to determine relative changes in expression levels of target genes. Design, assumptions and analysis of this method have been previously described by Livak, and Schmittgen (2001) and is an approach used extensively in the laboratory.

2.8.4.1 Selection of internal control; housekeeping genes

When using the 2 $^{-\Delta\Delta ct}$ method, it is important to apply a normalisation strategy. Housekeeping genes are commonly used as internal control, in order to normalise the qPCR data for the amount of RNA that was added to the reactions (Livak & Schmittgen, 2001). Internal control genes are genes regarded to be crucial for cell survival and thus ubiquitously expressed, with low variability (Thellin et al., 1999). Throughout the current thesis, three housekeeping genes were used: *Hprt*, *dynein*, and *β-actin* (Huggett, 2005; Isles, et al., 2004). Values obtained from these three genes were averaged to provide one single value per sample.

2.8.4.2 Data analysis

The change in gene expression levels were obtained in Ct values (defined as the number of cycles required for the fluorescence to exceed the threshold). Ct values were subsequently transferred to an Excel spreadsheet for conversion to $2^{-\Delta\Delta ct}$ and further analysis. For each sample, duplicates or triplicates of the Ct values were averaged. This averaged Ct value was subtracted from the averaged Ct value of the housekeeping genes to produce the ΔCt value. The next step was to calculate the ΔΔCt values by subtracting the ΔCt values of the experimental group from the ΔCt values of the un-treated/control group. The negative value, -ΔΔCt, was then used as the exponent of 2 (based on assumption that the reaction doubles the amount of product per cycle), and represented the "correct" number of cycles to the threshold (Livak & Schmittgen, 2001; VanGuilder, Vrana, & Freeman, 2008). Finally, -ΔΔCt values were presented as a graph.

2.9 General data presentation and statistical methods

Data were presented routinely as mean values ± standard error of the mean (SEM), using the following formula:

$$\text{SEM} = \text{standard deviation of values} / \sqrt{\text{number of values}}$$

Statistical analyses were performed using SPSS 16.0 for Windows (LEAD Technologies Inc., Apache Software Foundation, U.S.A.). Data were screened for normal distribution (Kolmorgorov-Smirnov test) prior to further analysis. Any variables that were significantly skewed or displayed kyrtosis were transformed using log10 transformation to ensure normal distribution. Data were analysed by independent Student's T test, two-way analysis of variance (ANOVA), or repeated measures analysis of variance (ANOVA). *Post hoc* comparisons were performed using Bonferroni correction. Greenhouse-Geisser degrees of freedom (df) corrections were applied, when necessary, to repeated measures ANOVA. For data deviating from normality, or violating the homogeneity of variance assumption (Levene's test) the relevant non-parametric tests were used (Mann Whitney U-test, Kruskal Wallis). When a correlation design was applied, Pearson's *r* correlation coefficient was used

(Spearman's *r*, if non-parametric test was in need). For all statistical comparisons, p values of ≤ .05 were regarded as significant. See individual experimental chapters for further details of specific statistical designs.

Chapter III

Initial physiological and behavioural characterisation of the 'four core genotype' (FCG) model

3.1 Introduction

The majority of the experimental work in this thesis focussed on the 'four core genotype' (FCG) model which, as was detailed in the General Introduction has some utility to dissociate between effects mediated by Y-chromosome linked *Sry* (both the well-established 'indirect' effects via gonadal hormones and the more recently appreciated 'direct' effects) and other sex chromosome linked genes. In terms of brain and behaviour, a number of reports have been published using the FCG model. Behaviours that have been investigated so far include male copulatory behaviour, pup retrieval and parenting, social exploration behaviour, aggression, nociception and aspects of cognition (Arnold & Chen, 2009; DeVries, et al., 2002; Gatewood, et al., 2006; Gioiosa, et al., 2008; Quinn, et al., 2007). The model has also been used to determine the extent to which *Sry* and other sex chromosome linked genes influence the sexually dimorphic development of neurochemical systems, including vasopressin-immunoreactive (ir) fibers in the lateral septum, tyrosine hydroxylase-ir neurons in the AVPV (antero-ventral paraventricular nucleus) and midbrain, progesterone receptor expression, and general gene expression in the brain (Carruth, et al., 2002; DeVries, et al., 2002; Wagner, et al., 2004).

As was noted earlier, some of these previous studies have used mice bred to an MF1 strain background (DeVries, et al., 2002; Quinn, et al., 2007), whilst others have used mice bred to a C57BL/6J background (Gatewood, et al., 2006; McPhie-Lalmansingh, et al., 2008). In this thesis, we used FCG mice on an MF1 background. Furthermore, in several of the earlier experiments with the FCG model, including the studies referenced above, mice were gonadectomised prior to testing in order to ensure that all had equivalent systemic gonadal hormonal levels. However, in this thesis, mice were not gonadectomised, as we wanted to avoid causing potential post-operative stress and discomfort, which might itself be influenced by *Sry*/sex chromosome-linked gene function, and also because we were interested in the

organisational and activational effects of gonadal hormones on brain function and behaviour *per se*.

In view of the different ways in which the FCG model may be utilised it was considered important, at the outset, to characterise the FCG model as used in our hands. Hence, this first experimental chapter was concerned with an initial physiological and behavioural specification of the FCG model with the purpose of: (i) confirming previous data obtained in the laboratory, (ii) extending the behavioural analysis into areas that hadn't yet been investigated and which were suspected to be potentially sexually dimorphic, (iii) where effects could be interpreted as '*Sry*-dependent', to begin to attempt to dissociate between indirect and direct effects, (iv) to gain information that may have alerted us to potential confounds in later experiments examining more focused components of behaviour.

Previously, Lynn (2010) observed that FCG mice on a MF1 background and maintained within the School of Psychology at Cardiff University showed low levels of mortality/morbidity and that all four genotypes were generated equally. We attempted to confirm this finding, and in addition examined bodyweight gain of the four genotypes over time as an index of general development and health. Lynn (2010) also found evidence for effects on activity levels in the FCG model. This finding was followed up here in two different behavioural paradigms. First, in a comprehensive screening assay, locomotor activity and a number of ancillary behaviours were monitored continuously over a 24 hour-period using automated 'phenotyper' cages (lights on for 12 hours, followed by lights off for 12 hours). As well as overall levels of activity this assay provided a number of additional informative measures including an index of reactivity to a novel environment and data indicating possible effects on circadian behaviours (De Visser, Van Den Bos, & Spruijt, 2005; Robinson, McKillop-Smith, & Riedel, 2008). The automated temporary homecages were further equipped with a running wheel that allowed investigation of the reported sex differences in this behaviour (where female rodents have been shown to be more active (Lightfoot, et al., 2004; Roper, 1976; Tokuyama, Saito, & Okuda, 1982); another advantage of the automated apparatus was that they provided data on consummatory (feeding/drinking) behaviours within the 24 hour period.

By way of obtaining a more direct comparison with the data of Lynn (2010), in a second behavioural paradigm locomotor activity was monitored across the four genotypes over an one hour period (during the light phase) using the identical bank of

activity cages used by Lynn previously. Specifically, Lynn (2010) employing the same statistical design as used in the present thesis reported a significant SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT, whereby only one of the four genotypes of the FCG model, the XY- mice were significantly and consistently more active than the other three genotypes. Confirmation of these data was of particular interest given XY- mice have also exhibited a trend to be more active in other circumstances by others, including the open field arena, commonly used to assay exploration/anxiety behaviours (McPhie-Lalmansingh, et al., 2008).

Two new lines of investigation were attempted in this chapter. The first was motivated by evidence that sex chromosome linked genes may influence aggression and social dominance behaviours in rodents and humans (Maxson, 1996; Roubertoux et al., 1994; Shoaib Shah, Ayub, Firasat, Kaiser, & Mehdi, 2009) and the well-established observation that male and female mice differ in their aggression/dominance profiles (Edwards, 1969; Gatewood, et al., 2006). FCG mice were assayed in the 'tube test' which has been validated as an index of social dominance, where pairs of mice confront each other in a narrow tube (in which it is not possible to turn round) and assessed as to which of the pair backs down first, allowing the dominant 'winner' passage to the other end of the tube. This task has been used routinely in the laboratory and shown to be sensitive to a range of genetic manipulations (Garfield et al., 2011; Lindzey, Winston, & Manosevitz, 1961; Trent et al., 2011). A second new line of investigation was to attempt to correlate any physiological/behavioural effects that appeared to be *Sry*-dependent with measures of systemic testosterone levels and *Sry* expression in brain; this was done as a means of attempting to distinguish between likely indirect and putative direct actions of *Sry* on function.

3.2 Materials and Methods

3.2.1 Generation of FCG mice

General information on the generation of FCG mice can be found in the Chapters I and II. Briefly, FCG mice were generated by pairing a XX female with a XY-*Sry* male. The Y- chromosome is a variant of the Y^{129}, deleted for the *Sry* gene (Tdy^{m1} mutation; (Lovell-Badge & Robertson, 1990). XY-*Sry* mice were initially made on an MF1 background by introducing a fully penetrant *Sry* transgene into pronuclear stage embryos of XY- females (XY^{tdym1})[3](Mahadevaiah, et al., 1998). By pairing XY-*Sry* males with XX females, we obtained the four different genotypes: XX, XY-, XY-*Sry*, and XX*Sry*. Immediately prior to weaning, all mice were initially distinguished by their external genitalia; following weaning, they were genotyped for definitive identification following the protocol outlined in Chapter II, section 2.5.

3.2.2 Subjects and animal husbandry

Details on general handling and housing conditions can be found on Chapter II, sections 2.1.1 and 2.2. Table 3.2.2i provides information on the number of animals used to monitor body weight and numbers used in each behavioural task. Behavioural testing commenced from three months of age.

Table 3.2.2i: Number of subjects used for body weight determinations and behavioural testing. Number in brackets denote original number before exclusion (more details on exclusion criteria in following sections)

	40, XX	40, XY-	40, XX*Sry*	40, XY-*Sry*
Body weight	15	15	8	15
Phenotyper cages	9 (10)	10	8	9 (10)
Locomotor activity	15	15	8	15
Social dominance tube test	15	15	8	8

[3] Y^{tdym1}mutation is a 14kb deletion in the short arm of the Y chromosome removing *Sry* (Lovell-Badge & Robinson, 1990).

3.2.3 Physiological measurements

3.2.3.1 Mortality, genotype distribution and general health

Early mortality rates were determined by counting litter size on postnatal day one, and then by daily monitoring until weaning (c.28 days). Following weaning, pups had their hair plucked for genotyping (following the protocol described in Chapter II, section 2.5), to allow the distribution of genotypes to be assessed and for subsequent experimental group allocation. In total 162 pups were examined; these came from 24 litters by 19 different mothers. General health of all animals was monitored regularly throughout the testing period.

3.2.3.2 Body weight

After weaning at 28 days, animals were weighed weekly for 14 weeks under conditions of *ad libitum* (*ad lib*) access to food and water.

3.2.3.3 Oestrous cycle

Vaginal smearing was performed daily during behavioural testing. Details on the protocol used can be found in Chapter II, section 2.2.4.

3.2.4 Behavioural measurements

3.2.4.1 24 hour continuous activity monitoring in Phenotyper cages

A description of the 'Phenotyper' apparatus for extended behavioural monitoring can be found in Chapter II, section 2.3.1.1. Each cage was equipped with infra-red sensitive cameras to allow detection of movement in the dark. Animals were placed in the cages (one mouse per cage) between 7:00 and 8:00 am and were allowed to freely explore and interact with the apparatus for 24 hours. During testing, the mice were maintained on a 12 hours light/dark cycle with lights going off between 7:00 and 8:00 pm. Water and food was available *ad lib*. Behavioural parameters that were recorded included locomotor activity in the main arena, frequency of entries and duration of

time spent in the shelter (the latter used as a proxy for time spent sleeping), frequency and duration of time spent on the running wheel, and distance travelled. Behavioural analysis was analysed in three hour time bins (therefore 8 bins in total). In addition, weight of food hopper (food consumption) and weight of water bottle (water consumption) were recorded at the beginning and end of each testing session. Between runs, the boxes were thoroughly cleaned with 1% acetic acid.

3.2.4.2 Activity cage battery

Motor activity was further assessed using dedicated locomotor activity chambers (see Chapter II, section 2.3.1.3). Animals were placed singly in the chambers and were free to explore for an hour. All subjects underwent one test session conducted under dim lighting conditions (~5 lux). The number of infra-red beam breaks was recorded by a computer running with BBC BASIC V6 programmes on the ARACHNID system (Cambridge Cognition Ltd., Cambridge, UK.). Between runs, the boxes were thoroughly cleaned with 1% acetic acid.

3.2.4.3 Tube test assay for social dominance

Social dominance was assayed using the tube test (Lindzey, et al., 1961). A description of the apparatus can be found in Chapter II, section 2.3.1.2. All animals underwent one session, which consisted of three trials. XX mice (n = 15) were always paired against XY- mice (n = 15), and XX*Sry* mice (n = 8) against XY-*Sry* mice (n = 8); hence, for the XX vs. XY- comparison, there were 45 trials in total, and for the XX*Sry* vs. XY-*Sry* comparison there were 24 trials in total. In this paradigm, we could only test for the presence/absence of a SEX CHROMOSOME COMPLEMENT effect on social dominance (as it was important to arrange that mice of the same gonadal sex were competing against each other). As far as possible, paired animals were matched for bodyweight. At the beginning of each trial, paired animals were placed at the opposite ends facing the tube and released simultaneously by the experimenter. The trial ended when one mouse was completely ousted from the tube. If, after two minutes, neither mouse had succeeded in removing its opponent from the tube, the trial was ended and a draw result was noted. After each trial, the mice were alternated in the direction they were placed into the tube.

3.2.5 RNA extraction and cDNA synthesis

A subset of the male mice (XY-*Sry*: n = 12; XX*Sry*: n = 8) were sacrificed by cervical dislocation and hemi-brain tissue was dissected on ice (Chapter II, section 2.7). RNA was extracted from homogenised tissue (at 4ºC in a FastPrep FP120 micro-homogenizer; MB Biomedicals, U.S.) in accordance with standard protocols (Chapter II, section 2.8.1). RNA quality and concentration were assessed with the use of a spectrophotometer (NanoDrop® ND-1000 UV-Vis, Wilmington, DE). For each RNA sample, measurement of the optical density at 260 and 280 nm was taken. All samples were subsequently DNase treated, in order to remove residual DNA (Ambion). 1µg RNA was converted to cDNA using a Sprint™ RT Complete Products Kit (Clontech, Mountain View, CA) and random hexamer primers.

3.2.6 Gene expression analysis

Real time qPCR was performed using a Rotor-Gene™6000 cycler machine. A Corbett CAS-1200 robotic bench top instrument (Corbett Life Science) was used for automated PCR setup. Each sample (15ul volume) included 5ul of diluted cDNA sample, 0.3ul primers (forward and reverse), 1.9ul of H_2O, and 7.5ul of 2X SensiMix SYBR (Quantace). All samples were tested in duplicate to eliminate pipetting errors. PCR cycling conditions and primer sequences have been described on Chapter II, section 2.8.3). Applying a normalisation strategy, when qPCR method is used, is of importance, as discrepancies and false data can be generated due to sample variability (Bustin, 2005). Therefore, so-called 'housekeeping' control genes are commonly used; the expression of these genes should be ubiquitous and similar among the experimental samples (VanGuilder, et al., 2008). Here, three commonly used housekeeping genes were used for normalisation: *Gapdh*, *Dynein*, and *β-actin* (Huggett, 2005; Isles, et al., 2004). For each sample, Ct values obtained from the three genes were averaged to provide a single normalisation value. Table 3.2.6i provides sequences for all genes. The Ct values obtained were analysed using the $2^{-\Delta\Delta CT}$ Method, as described in Chapter II, 2.8.4.2. Transformed ΔCt values, $2^{-\Delta Ct}$, were used for data analysis, as they are linear (contrary to exponential Ct values; greater $2^{-\Delta Ct}$ values denote greater gene expression).

Table 3.2.6i: Primer sequences of housekeeping and target genes used in this chapter

Gene	Primer direction	Primer sequence
Gapdh (h/keeping)	Forward	5’GAACATCATCCCTGCATCCA3’
	Reverse	5’CCAGTGAGCTTCCCGTTCA3’
Dynein (h/keeping)	Forward	5’GGACATTGCTGCCTATATCAAGAAG3’
	Reverse	5’CGTGTGTGACATAGCTGCCAA3’
B-actin (h/keeping)	Forward	5’TCTGTGTGGATTGGTGGCTCTA3’
	Reverse	5’CTGCTTGCTGATCCACATCTG3’
Sry (target)	Forward	5’TTTCCAGGAGGCACAGAGAT3’
	Reverse	5’GCAGGCTGTAAAATGCCACT3’

3.2.7 Testosterone serum levels analysis

Upon culling, trunk blood was collected from a subset of FCG male mice (XY-*Sry*: n = 14; XX*Sry*: n = 7). Details on blood collection, serum storage and testosterone analysis can be found in Chapter II, section 2.6.

3.2.8 Statistical analysis

Data were presented as means ± standard error. Analysis was performed using Two Way ANOVA with SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT as factors; where necessary, a third within-group factor of TIME BIN was used. Data that deviated from normality (according to the Kolmogorov-Smirnov test) were subjected to appropriate transformation in an attempt to normalise; if data were still not normal, they were analysed by Mann-Whitney U test. The social dominance tube test and genotype distribution data were analysed by chi-squared test. Correlational analysis was conducted using Spearman’s coefficient, as data deviated from normality (Kolmogorov-Smirnov test). When necessary, Bonferroni correction was applied to correct for multiple testing. P values ≤ .05 were regarded as significant.

3.3 Results

3.3.1 Physiological data

3.3.1.1 Mortality, genotype distribution and general health

Continuous monitoring of the FCG animals did not reveal any serious health problems across the four genotypes. Throughout the course of these studies, four litters were born dead (all pups died). In total, 162 pups from the FCG cross were born alive (average litter size 6.75), with all but two pups surviving up to weaning (mortality rate < 1.5%). There was no significant difference in actual genotype distribution from the anticipated mendelian distribution (i.e. 25% of each genotype) (χ^2 (3, N=160) = 5.750, p= .124) (Table 3.3.1.1i).

Table 3.3.1.1i: Genotype distribution for FCG progeny to weaning

	XX	XY-	XX*Sry*	XY-*Sry*
Number of pups	52	38	31	39

3.3.1.2 Body weight

As shown in Figure 3.3.1.2a, all mice gained weight between weaning and postnatal week 14 as expected (effect of TIME BIN, $F_{3.325,\ 162.901}$=198.255, p< .001), with gonadal males consistently being heavier than gonadal females (effect of SRY DEPENDENCE; $F_{1,49}$=14.029, p< .001). There was no main effect of SEX CHROMOSOME COMPLEMENT ($F_{1,49}$= .257, p= .615) nor a significant interaction between the two factors ($F_{1,49}$= .000, p= .990).

Figure 3.3.1.2a

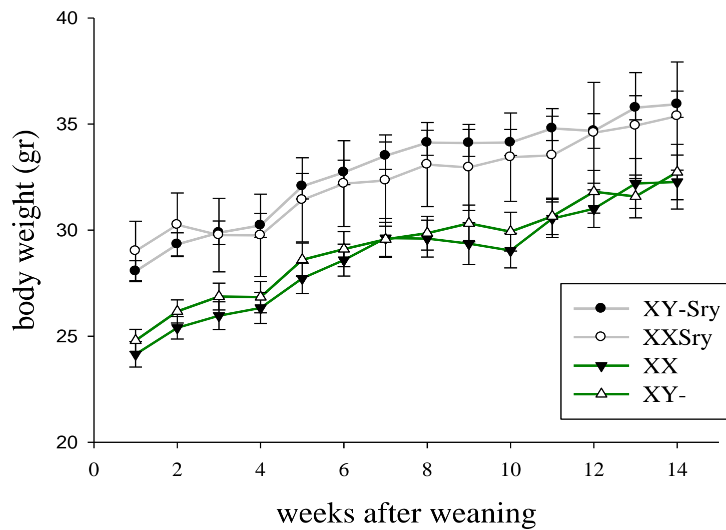


Figure 3.3.1.2a: Body weight of experimental subjects from weaning onwards. Gonadally male mice were heavier from one week post weaning until young adulthood, than gonadally female mice.

3.3.2 Behavioural data

3.3.2.1 24 hours continuous monitoring in phenotyper cages

For two animals (one XX and one XY-*Sry*), data was not recorded for subsequent analysis due to technical problems. Thus, the total number of experimental subjects used for analysis was 36 (XX = 9, XY- = 10, XX*Sry* = 8, XY-*Sry* = 9). Due to skewness of data, statistical analyses were conducted using log_{10} transformed data. On the main measure of distance travelled measured by continuous tracking across the arena, there were significant main effects of TIME BIN and SRY DEPENDENCE ($F_{2.682,\ 85.836}$=48.067, p< .001; $F_{1,32}$=10.682, p< .01, respectively), consistent with the expected greater activity during the dark phase in all genotypes, and with greater general activity in gonadal females than in gonadal males across the whole test period (Figure 3.3.2.1a). There were no significant interactions between any of the factors tested in this analysis (Table 3.3.2.1i).

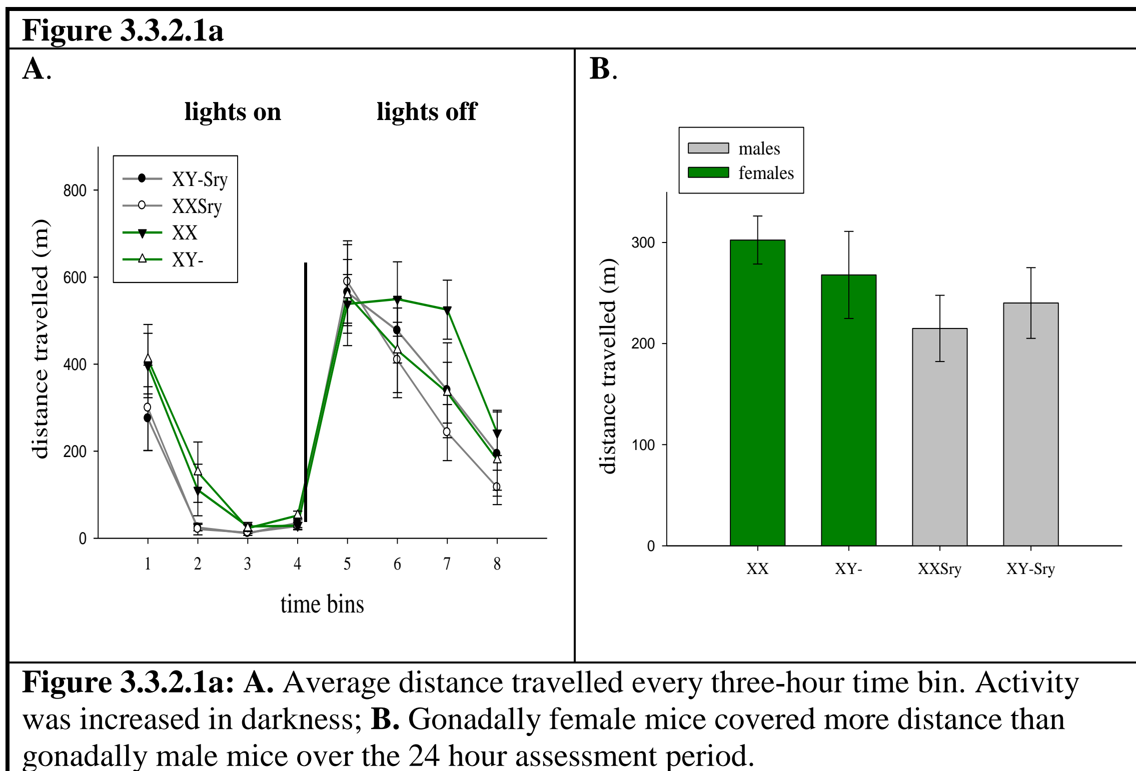


Figure 3.3.2.1a: A. Average distance travelled every three-hour time bin. Activity was increased in darkness; **B.** Gonadally female mice covered more distance than gonadally male mice over the 24 hour assessment period.

Table 3.3.2.1i: Summary statistics for distance travelled in phenotyper cage analysis

	SRY DEPENDENCE	SEX CHROMOSOME COMPLEMENT	SRY DEPENDENCE *SEX CHROMOSOME COMPLEMENT	TIME* SRY DEPENDENCE	TIME*SEX CHROMOSOME COMPLEMENT
Distance (cm)	$F_{1,32}$=10.682, p< .01	$F_{1,32}$= .029, p= .867	$F_{1,32}$= .468, p= .499	$F_{2.682,85.836}$= 2.146, p= .107	$F_{2.682,85.836}$= 1.045, p= .371

Analysis of an additional measure of activity, number of revolutions in the running wheel, revealed significant effects of TIME BIN ($F_{3.549,\ 110.030}$= 30.376, p< .001), again consistent with the mice generally being more active during the dark phase of testing (Figure 3.3.2.1b). However, there were no main effects of SRY DEPENDENCE or SEX CHROMOSOME COMPLEMENT on this measure, nor any significant interaction between SRY DEPENDENCE, SEX CHROMOSOME COMPLEMENT and TIME BIN (Table 3.3.2.1ii).

Figure 3.3.2.1b

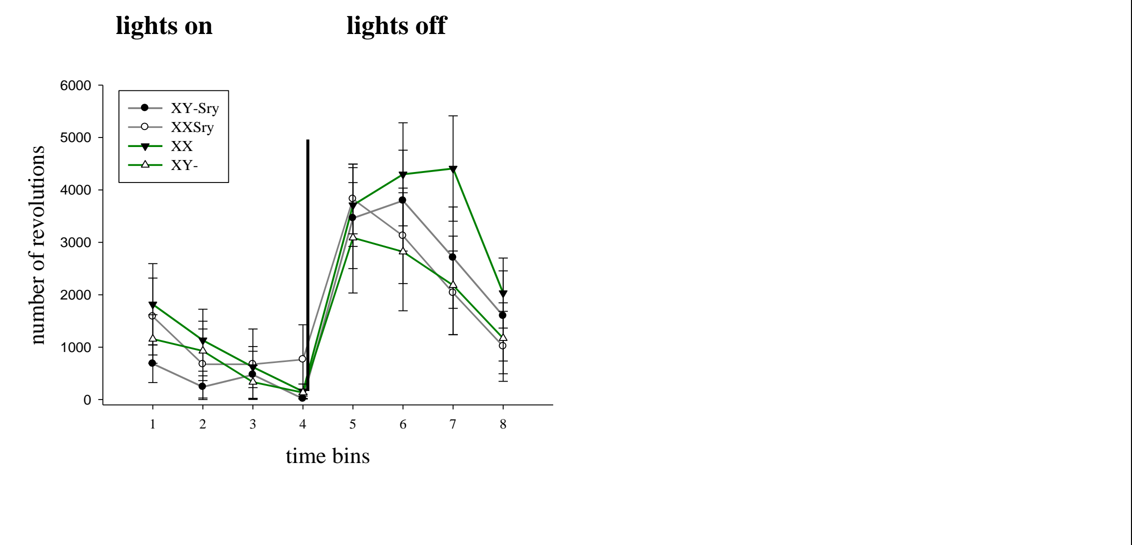


Figure 3.3.2.1b: Number of revolutions on the running wheel. All mice were more engaged during the dark period compared to light.

Table 3.3.2.1ii: Summary statistics for running wheel analysis

	SRY DEPENDENCE	SEX CHROMOSOME COMPLEMENT	SRY DEPENDENCE *SEX CHROMOSOME COMPLEMENT	TIME BIN* SRY DEPENDENCE	TIME*SEX CHROMOSOME COMPLEMENT
Number of revolutions on running wheel	$F_{1,31}$= .269, p= .608	$F_{1,31}$= .604, p= .443	$F_{1,31}$=1.894, p= .179	$F_{3.549,110.030}$= 1.858, p= .131	$F_{3.549,110.030}$= .545, p= .682

Time spent in the shelter was recorded as a proxy measure of time spent sleeping. ANOVA revealed a significant effect of TIME BIN on duration of time spent in the shelter ($F_{4.504,\ 144.141}$=16.696, p< .001), consistent with the mice sleeping during the light phase, and being more active in the dark phase (Figure 3.3.2.1c). There were no statistically significant effects of SRY DEPENDENCE or SEX CHROMOSOME COMPLEMENT on this measure, nor any significant interactions between the three factors of SRY DEPENDENCE, SEX CHROMOSOME COMPLEMENT and TIME BIN after accounting for multiple testing (Table 3.3.2.1iii).

Figure 3.3.2.1c

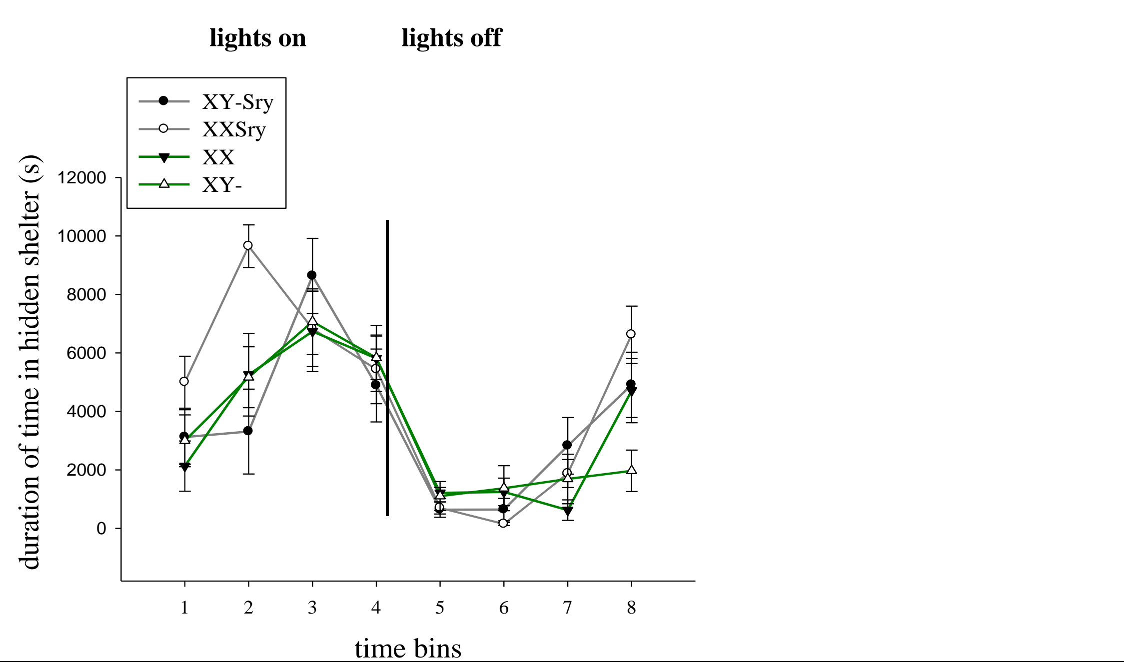


Figure 3.3.2.1c: Duration of time spent in the hidden shelter. All mice made less entries and spent less time in the shelter during the dark period.

Table 3.3.2.1iii: Summary statistics for shelter analysis

	SRY DEPENDENCE	SEX CHROMOSOME COMPLEMENT	SRY DEPENDENCE *SEX CHROMOSOME COMPLEMENT	TIME BIN* SRY DEPENDENCE	TIME*SEX CHROMOSOME COMPLEMENT
Duration in shelter	$F_{1,32}$=.002, p= .967	$F_{1,32}$=1.422, p= .242	$F_{1,32}$= .052, p= .820	$F_{4.504,144.141}$=2.471, p< .05 (non-significant after Bonferroni correction)	$F_{4.504,144.141}$= .635, p= .657

Analysis of food and water consumption was conducted on data normalised for body weight at the time of testing (consumption/body weight$^{0.75}$)(Doe et al., 2009). XY chromosome carriers consumed significantly more food than XX chromosome carriers within the 24 hour test period (effect of SEX CHROMOSOME COMPLEMENT, $F_{1,31}$=12.248, p< .01); in addition, there was a significant effect of SRY DEPENDENCE ($F_{1,31}$=5.659, p< .05), whereby gonadal male mice consumed more food than gonadal female mice (Figure 3.3.2.1d). No significant interactions were observed. Analysis of water consumption did not reveal any main effects of SRY DEPENDENCE or SEX CHROMOSOME COMPLEMENT, or any interaction between the two factors (Figure 3.3.2.1d and Table 3.3.2.1iv).

Figure 3.3.2.1d

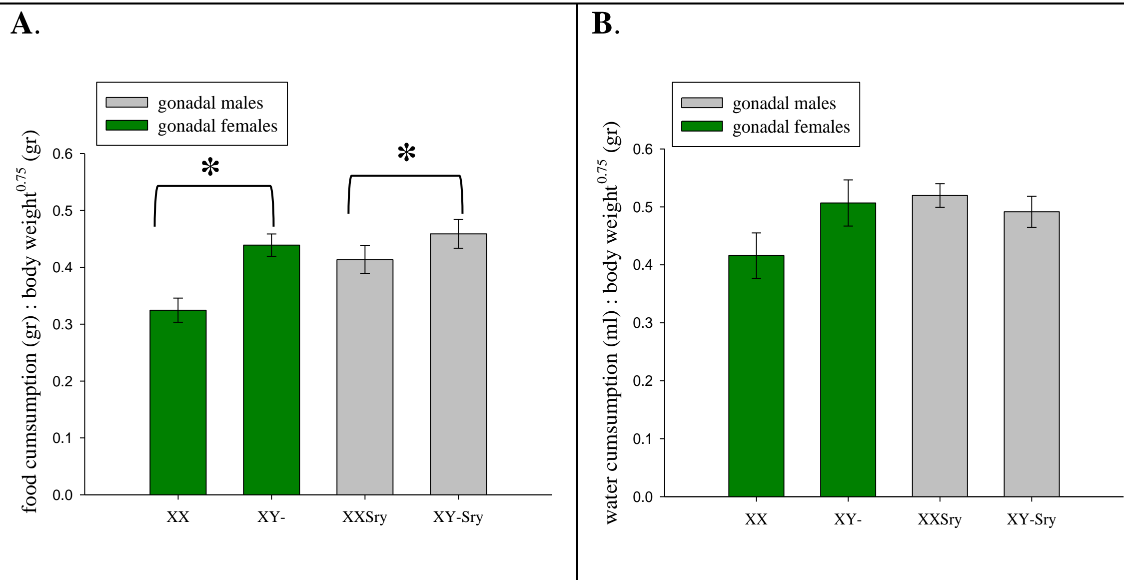


Figure 3.3.2.1d: After controlling for body weight and adjusting for metabolic scaling, XY carriers consumed more food than XX carriers; * $p < .01$ (Least Significant Differences/LSD test for pair-wise comparisons); additionally, gonadal male mice consumed significantly more food than gonadal female mice (**A.**), there were no significant differences in the data for water consumption (**B.**)

Table 3.3.2.1iv: Summary statistics for food and water consumption analysis

	SRY DEPENDENCE	SEX CHROMOSOME COMPLEMENT	SRY DEPENDENCE *SEX CHROMOSOME COMPLEMENT
Water consumption	$F_{1,32}=1.7$, $p= .202$	$F_{1,32}=.857$, $p= .362$	$F_{1,32}=3.085$, $p= .089$
Food consumption	$F_{1,31}=5.659$, $p< .05$	$F_{1,31}=12.248$, $p< .01$	$F_{1,31}=2.274$, $p= .142$

Table 3.3.2.1v: Summary table of significant main effects of SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT in phenotyper cage analysis

Factor	Behavioural parameter
SRY DEPENDENCE	1. Distance travelled: gonadal female mice covered a greater distance during 24 hours testing than gonadal male mice. 2. Food consumption: gonadal male mice ate more than gonadal female mice over a 24 hour period.
SEX CHROMOSOME COMPLEMENT	1. Food consumption: XY carriers consumed more food than XX carriers over a 24 hour period.

3.3.2.2 Activity cage battery - 1 hr test

There was no significant effect of SRY DEPENDENCE on the main measure of activity in the activity cages (i.e. total infra-red beam breaks) in the 1 hr test session. However, consistent with the results from the more extended measure of locomotor activity determined in the phenotyper cages, there was a strong trend for gonadal female mice to be more active than gonadal male mice (effect of SRY DEPENDENCE; $F_{1,49}$=3.060, p= .08) (Figure 3.3.2.2a). There were no other main effects or interactions in the data (SEX CHROMOSOME COMPLEMENT; $F_{1,49}$= .745, p= .392; SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT; ($F_{1,49}$= .010, p= .919).

Figure 3.3.2.2a

A.

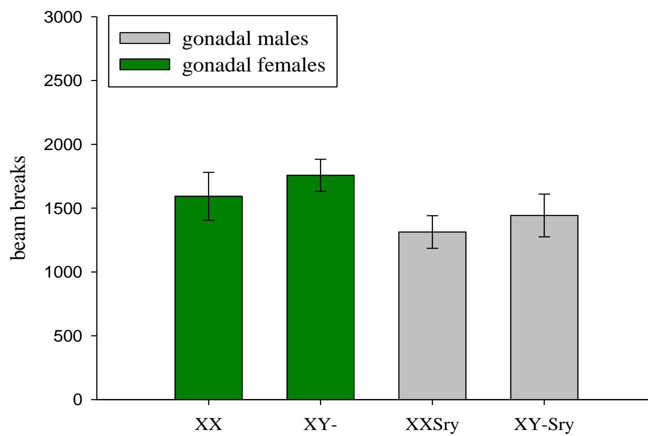


Figure 3.3.2.2a: Total beam breaks during a 1 hr locomotor activity session. Gonadal females displayed a tendency to be more active than gonadal males, albeit non-significant.

3.3.2.3 Social dominance tube test

Of the 45 trials in which XX and XY- mice were paired, five were drawn. Of the trials which had a winner, there was no significant difference in frequency of wins between XX and XY- mice (XX mice won 15, and XY- mice won 25; $\chi^2(1)=1.27$, p= .260). Of the 24 trials in which XX*Sry* and XY-*Sry* mice were paired, four were drawn. Of the trials which had a winner, XX mice did not win significantly more frequently than XY- mice (XX mice won 9, and XY- mice won 11; $\chi^2(1)= .100$, p= .752).

3.3.3 Co-variance with stage of oestrous

For behaviours in the gonadally female mice where there were significant effects involving factor SRY DEPENDENCE an analysis of covariance (ANCOVA) was performed, with GENOTYPE (XX and XY-) as the fixed factor and OESTROUS STAGE as the covariate, in order to test for oestrous-stage related effects on behaviour. The analysis did not yield any significant effect of OESTROUS STAGE (see Appendix A). Therefore, using these physical measures as a proxy for hormonal status, it did not appear that stage of oestrus influenced any behavioural effects observed in the gonadally female mice.

3.3.4 Brain Sry expression and serum testosterone levels; correlation with Sry-dependent measures

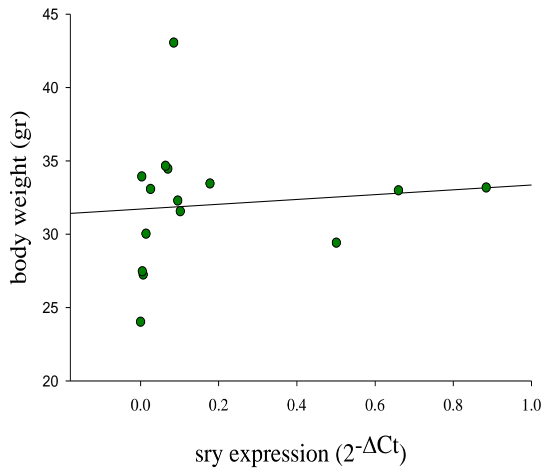
A correlational analysis was attempted matching individual levels of hemi-brain *Sry* expression and serum testosterone levels in XY-*Sry* and XX*Sry* mice with the following physiological/behavioural *Sry*-dependent parameters: body weight averaged across experimental period, total distance travelled and food consumed over 24hr in the phenotyper cages. Bonferroni correction was applied to correct for the six different correlations (i.e. $p \leq .008$ was regarded as significant). Table 3.3.4i shows the average values for *Sry* brain expression and testosterone levels yielded in this analysis. *Sry* expression levels were similar to those reported previously by our lab (P. M. Y. Lynn, 2010). In terms of testosterone, present values were a bit higher than those in Lynn (2010) (mean value of XX*Sry*: 0.4±0.09 (ng/ml); mean value of XY-*Sry*: 1.03±0.4 (ng/ml)). However, the present testosterone serum levels are within the range expected for male mice (DeVries, et al., 2002; Gatewood, et al., 2006; Samuel Barkley & Goldman, 1977; Svare et al., 1983).

Table 3.3.4i: Mean±SEM values for hemi-brain *Sry* expression and trunk blood testosterone levels

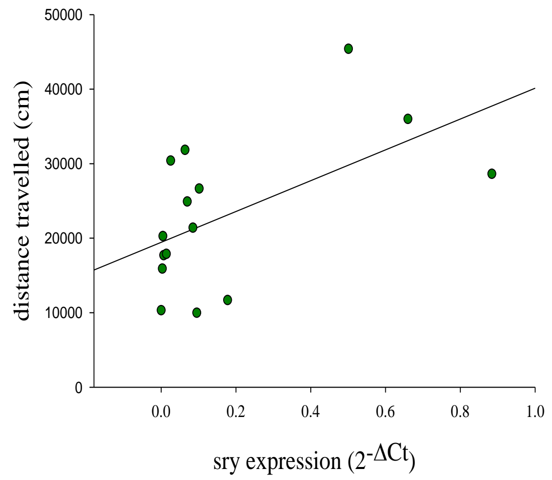
	Mean	SEM
Sry	0.18 ($2^{-\Delta Ct}$; relative quantity values)	0.075
Testosterone	3.26 (ng/ml)	1.11

Figure 3.3.4a

A.



B.



C.

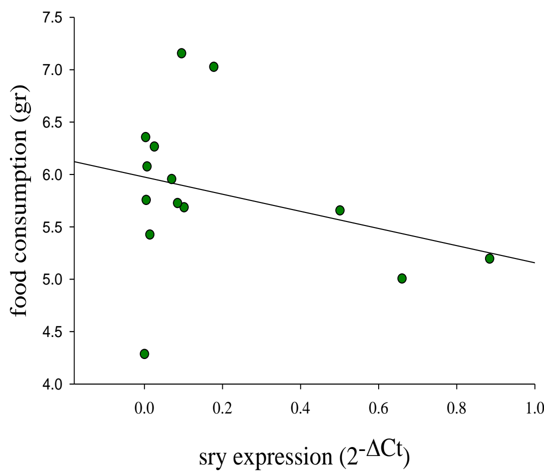


Figure 3.3.4a: Correlation between Sry expression levels (expressed as relative quantity values) in individual hemi-brain samples and the following physiological and behavioural measurements: body weight (**A.**), distance travelled (**B.**), and food consumption (**C.**).

Figure 3.3.4b

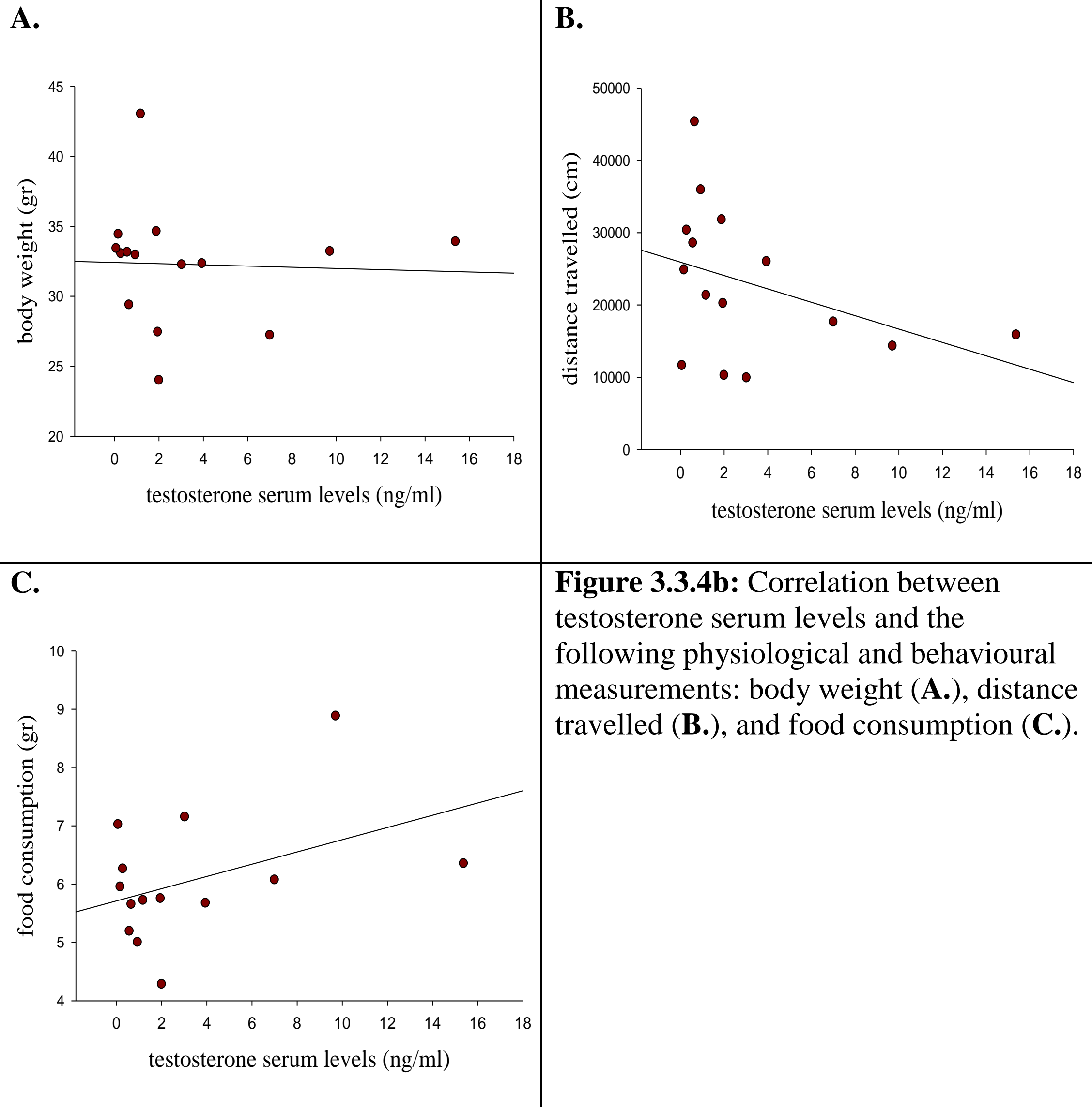


Figure 3.3.4b: Correlation between testosterone serum levels and the following physiological and behavioural measurements: body weight (**A.**), distance travelled (**B.**), and food consumption (**C.**).

Figures 3.3.4a and Figure 3.3.4b show the relationships between the *Sry*-dependent measures of interest (after adjusting for multiple testing) and hemi-brain *Sry* expression or serum testosterone levels, respectively. Analysis by Spearman's coefficient did not reveal any significant correlations, though the correlation between brain *Sry* expression and distance travelled over 24 hr in the phenotyper cages was close to being nominally significant ($p<0.06$) (Table 3.3.4ii).

Table 3.3.4ii: Summary of statistics for correlational analyses

Physiological/behavioural measure	*Sry* expression levels	Testosterone serum levels
Body weight	r = .286, p= .302	r = -.264, p= .341
Distance travelled	r = .500, p= .058	r = -.418, p= .121
Food consumption	r = -.160, p= .584	r = .222, p= .446

3.4 Discussion

Mice of all four genotypes appeared, on general observation, to be healthy and no serious health issues arose during the experimental period. Consistent with previous findings (Lynn, 2010), we did not find any divergence from the expected genotype distribution at weaning, implying no significant differences in mortality between the genotypes *in utero* or in the early postnatal period. In general, FCG mice displayed basic physiological and behavioural profiles similar to those of inbred and outbred (MF1) mice assayed on the same tests within our laboratory over several years.

A first statistically reliable finding was an effect of SRY DEPENDENCE on body weight, whereby gonadal males were heavier than gonadal females from weaning onwards; as mice were not routinely weighed prior to weaning it is not possible to comment on the temporal origin of this effect. In the temporary homecage 24 hr paradigm, we found a second effect of SRY DEPENDENCE on general activity, insofar as gonadal females were more active than gonadal males. As no significant effect of SRY DEPENDENCE was seen on time spent in the hidden shelter (a rough index of sleeping) it is likely that the difference in activity scores were not simply the result of increased time in the main arena but that gonadal females were, in fact, more active when they were in the main arena. These data are consistent with previous evidence from wild type rodents (both mice and rats) showing females to be more active than males (Blizard, Lippman, & Chen, 1975; Kas, De Mooij-van Malsen, Olivier, Spruijt, & Van Ree, 2008; Koos Slob, et al., 1986; D. A. Lynn & Brown, 2009; Tarantino, et al., 2000).

During the 24hr temporary homecage monitoring period, we also noted a significant effect of SRY DEPENDENCE on food consumption (after normalising for body weight), with gonadal males consuming more than gonadal females; these data are consistent with previous data showing greater consumption in wild type males than females (Bell & Zucker, 1971a; Koos Slob & Van Der Werff Ten Bosch, 1975a), and suggest that gonadal males do not consume more food simply because they are heavier. The greater body weight of gonadally male mice may be due to a combination of their increased food consumption and (speculatively), their greater degree of inactivity relative to gonadal female mice. An effect of SEX CHROMOSOME COMPLEMENT was found superimposed upon that of SRY DEPENDENCE, whereby mice possessing an XY karyotype consumed more food

than mice possessing an XX karyotype irrespective of gonadal status. This finding implies that there are one or more sex-linked genes other than *Sry* that might influence food consumption; one obvious candidate is the X-linked *Htr2c* gene (encoding the 2c subunit of the 5-HT receptor) which has been strongly associated with feeding behaviours in mice (Nonogaki, Strack, Dallman, & Tecott, 1998; Tecott et al., 1995; Vickers, Clifton, Dourish, & Tecott, 1999). A second candidate gene is *Mecp2*, an X-linked gene which, when deleted in the murine hypothalamus, causes hyperphagia (Fyffe et al., 2008). There were no significant main effects or interactions involving TIME BIN in the phenotyper cage data, implying no differential reactivity to novelty or differential basic circadian behaviours across the four genotypes.

In the shorter term (1 hr) locomotor activity cage assay, a trend towards increased activity in gonadal females was also found. However, under conditions that were very similar to those used previously, we did not replicate the data of Lynn (2010) showing markedly greater activity in XY- mice than the other three genotypes. The reasons for this discrepancy are unclear but the present data are supported by findings obtained by others using the FCG model, whereby activity in gonadal females in an open field test was elevated, irrespective of genotype (McPhie-Lalmansingh, et al., 2008).

In the present study, subjects were assayed in the tube test, an index of social dominance/aggression. Previous work by Gatewood and colleagues (2006) tested FCG mice (on a C57BL6/J background) in a resident/intruder paradigm and found that XY- mice were more aggressive, displaying behaviour similar to gonadal males (XX*Sry* and XY-*Sry)*, and not XX females, whilst a similar effect was also noted in another study using FCG model (McPhie-Lalmansingh, et al., 2008). Here, our analysis did not reveal any significant differences between the genotypes (XX vx XY, and XX*Sry* vs XY-*Sry*), although XY- mice did win more bouts than their XX counterparts. It could be argued therefore that there are no large sex chromosome effects on social dominance/aggressive behaviour, as measured by the tube test, in FCG mice. Nevertheless, it should be noted that the present study did not make use of all the available tests assaying social dominance in mice. In a recent paper, for example, Wang and colleagues (2011) reported five additional behavioural assays which are used to measure dominance in mice and correlate with performance in the tube test. Thus, one way forward would be to test XX and XY- carriers in the tube test, along with other behavioural assays, such as the visible burrow system (VBS)

and agonistic behaviour test (Wang et al., 2011), in order to achieve a more comprehensive picture of the role that sex chromosome complement could exert in this behavioural phenotype.

Sry-related effects (i.e. effects of SRY DEPENDENCE) on bodyweight, food consumption, and activity could be attributed either to a direct *Sry* effect on the brain, or alternatively could be mediated indirectly by hormonal secretions, notably testosterone. There is some evidence linking testosterone levels to bodyweight and food intake (animals with higher testosterone levels weigh and eat more) (Beatty, 1973; Bell & Zucker, 1971a; Koos Slob & Van Der Werff Ten Bosch, 1975a; Nunez, Siegel, & Wade, 1980), and other evidence showing that *Sry* can exert a direct, cell-autonomous effect on motoric function via effects on important circuits modulating motor output in the substantia nigra (Dewing, et al., 2006). A priori, the present study does not support a direct effect of either *Sry* or testosterone, as neither was shown to correlate with the aforementioned parameters. These negative results could be attributed to several factors, including the relatively small sample size, the possibility of a non-linear correlation between *Sry* expression/testosterone levels and physiological/behavioural measures, and the potential lack of specificity when measuring gene expression from whole brain rather than from discrete regions of high *Sry* expression.

3.4.1 Summary points

- The four genotypes within the FCG model were generated in equal proportions; there were no issues related to differential post-natal mortality rates or general health across the component genotypes.
- In the extended 24 hour assessment of behaviour in the 'phenotyper cages';
  - After accounting for body weight and metabolic scaling gonadally male mice (XY-*Sry*, XX*Sry*) were heavier, and consumed more food than gonadally female mice (XX, XY-); additionally, XY mice consumed more food than XX mice irrespective of gonadal status, these data suggesting independent effects of SRY DEPENDENCE in the first instance and SEX CHROMOSOME COMPLEMENT in the second.
  - Gonadally female mice (XX, XY-) were more active than gonadally male mice (XY-*Sry*, XX*Sry*) in terms of horizontal locomotor activity in the main area of the cage (but not in terms of wheel running).

- There were no other statistically reliable effects of genotype in any of the other measures, including indices related to circadian behaviours/sleep.

- In the 1 hour test in the battery of activity cages there were no statistically significant effects of genotype; this differed to previous data indicating higher activity in the XY- genotype in the same test and apparatus.
- There was no evidence for genotype effects on social dominance/aggressive behaviours as measured by the tube test.
- Effects of SRY DEPENDENCE could not be readily explained by *Sry* brain expression or serum testosterone levels.

Chapter IV

Anxiety-related behaviours in the 'four core genotype' (FCG) model

4.1 Introduction

A substantial body of evidence suggests that anxiety disorders are, in general, more prevalent among women than men; for example, women are more likely to be diagnosed with generalised anxiety disorder (GAD), panic disorder, agoraphobia, and post-traumatic stress disorder (PTSD) during adolescence and/or in adult life (Holden, 2005; McLean & Anderson, 2009). The sexually dimorphic prevalence of such disorders may result from various factors; genetic, hormonal, environmental and (possible) diagnostic ascertainment biases. Females possess a different sex chromosome complement to males (XX vs XY), have a different hormonal milieu (higher oestrogen and lower testosterone levels) and experience different social challenges and life events, all of which could contribute to a raised vulnerability to anxiety disorders in women. Moreover, such disorders may be differentially diagnosed between the sexes, creating a potential ascertainment bias, which could render the data difficult to interpret in terms of biological mechanisms (Hartung & Widiger, 1998; Potts, Burnam, & Wells, 1991).

Different experimental approaches have been used by researchers in an attempt to disentangle the biological mechanisms underlying sex differences in neuropsychiatric disorders, including anxiety-related behaviours. Such approaches include work in humans employing twin studies, clinical samples, neuroimaging and genome-wide and candidate gene association studies, as well as a large number of animal studies that have taken advantage of the enhanced experimental control and access to body tissues offered by animal models (Cosgrove, et al., 2007; Eckel et al., 2008; McGuffin et al., 2005; Shifman et al., 2008) (see also Table 4.1i).

Table 4.1i: Examples of studies on gender differences in anxiety-related mechanisms and disorders

Method	Evidence for sexual dimorphism	Reference
Review of genetic epidemiology on anxiety disorders	For generalised anxiety disorder a significant effect of common familial environment was evident only for women.	(Hettema, Neale, & Kendler, 2001)
Positron emission tomography (PET)	Harm avoidance was negatively correlated with glucose metabolism in the anterior vmPFC only in females, but not in males.	(Hakamata et al., 2009)
Psychophysiological measures (heart rate, blood pressure)	Women displayed greater heart rate reactivity and negative affect (as measured by the Positive and Negative Affective Scale/PANAS) after repeated exposure to laboratory stressor than men. They also reported more intrusive thoughts and avoidance.	(Schmaus, Laubmeier, Boquiren, Herzer, & Zakowski, 2008)
Review of gender differences on anxiety disorders	Women are at a greater risk to develop anxiety disorders and phobias due to various factors including differences in physiological reactivity, hormonal fluctuations, higher scores in negative affectivity and trait anxiety. Women also tend to ruminate and worry more, and are more sensitive to social cues than men.	(McLean & Anderson, 2009)

Some of the past literature on sex differences in anxiety in rodents has shown that, female rats display fewer anxiety-related behaviours than male rats in elevated plus maze (EPM) and open field tasks, assays which make use of the conflict between the natural instinct to explore new environments and the fear of exposure (Blizard, et al., 1975; Johnston & File, 1991; Koos Slob, et al., 1986; Xiang, Huang, Haile, & Kosten, 2011; Zimmerberg & Farley, 1993). Similar results have been obtained in studies using mice; notably, female mice spend more time in the open arms of the EPM and in the central area of the open field than male mice, a pattern of behaviour which is generally accepted to index reduced anxiety in females (Gioiosa, et al., 2007; Voikar, Koks, Vasar, & Rauvala, 2001). However, there are numerous cases that disagree with the general findings; for example, Chiba and colleagues showed that male mice displayed fewer anxiety-related behaviours than female mice in the EPM (Chiba, Matsuwaki, Yamanouchi, & Nishihara, 2009). These discrepancies could be explained by strain effects (An et al., 2011; Voikar, et al., 2001) and the fact that oestrus status in females, a potential mediator of hormone-mediated anxiety-related behaviours (Morgan & Pfaff, 2002; Toufexis, et al., 2006; Zimmerberg & Farley, 1993), is not always properly accounted for. In summary therefore, the extent to which sex differences impact on emotional behaviours is not a settled issue.

The main aim of the present chapter was to use the ‘four core genotype’ model to test the extent to which *Sry*, sex chromosome genes other than *Sry*, or a combination of the two, might influence anxiety-related phenotypes in mice, and to see whether these mechanisms could potentially influence sexually dimorphic anxiety-related behaviours. Previous work examining anxiety-related behaviours using this model has been inconclusive. Using mice on a C57BL/6J background strain, McPhie-Lalmansingh and colleagues (McPhie-Lalmansingh, et al, 2008) did not find any significant differences between the four different genotypes in terms of performance on the EPM and the open field arena. In contrast, work carried out within our own lab (P. M. Y. Lynn, 2010) using mice on an MF1 background has demonstrated a *Sry*-dependent effect on anxiety-related behaviour, whereby gonadally male mice displayed reduced anxiety-related behaviour than gonadally female mice on the zero maze (i.e. more time on the exposed open quadrants), irrespective of their sex chromosome complement. The ‘zero maze’ takes advantage of the same conflict between the drive to explore and the fear of exposure as the EPM but differs in that it does not have any central area leading on to the arms of the maze; this tightens up the

data insofar as the subjects can only be in either the closed (less aversive) or open (more aversive) parts of the maze. Hence, the discrepant results noted above could be explained by the different task used, strain effects, or by the fact that the mice in the former study were gonadectomised (i.e. gonads surgically removed at 2-4 months of age, to minimise hormonal activational effects), whereas the mice in the latter study were not.

This chapter sought to replicate and extend the findings previously obtained in our laboratory using larger experimental cohorts and several behavioural assays of anxiety, including the EPM. In this way, we could make more of a direct comparison with previous attempts by others to examine anxiety in the FCG model (P. M. Y. Lynn, 2010; McPhie-Lalmansingh, et al., 2008) and also look for converging (or diverging) evidence across a number of different tests for anxiety. This was important because anxiety-related behaviours are heterogeneous and multifaceted, and the available behavioural tasks are thought to assay different aspects of anxiety (Bourin, Petit-Demouliere, Dhonnchadha, & Hascoet, 2007). For this reason, FCG mice were tested on four different, but widely used, anxiety-related paradigms: the EPM, the elevated zero maze, the open field arena, and the light-dark box (Belzung & Griebel, 2001; Bourin & Hascoet, 2003; Prut & Belzung, 2003; Shepherd, Grewal, Fletcher, Bill, & Dourish, 1994; Wall & Messier, 2000).

4.2 Materials and Methods

4.2.1 Production of experimental subjects, animal husbandry and pre-test handling

In total, 89 FCG mice, aged 3-6 months, were used for this experiment. Mice were produced and housed at the Behavioural Neuroscience Lab of the School of Psychology, in Cardiff, as outlined in the General Introduction and Chapter II, 2.1.1. Table 4.2.1i shows the number of different genotypes used for this study. In addition, twenty-three normal, non-GM, MF1 male mice were tested in parallel. These mice were bred at the same lab in Cardiff, as the FCG mice, were of the same age and subjected to identical housing and general husbandry conditions (see also Chapter II, section 2.1.2). Two weeks prior to testing, all animals were subjected to daily handling (approximately 1 minute per animal per day).

Table 4.2.1i: Number of animals used in behavioural tasks; number in brackets denotes the number of animals included in analysis, when different from initial number (for further details, see sections 4.2.3.1 and 4.2.3.2).

Genotype	EPM	Zero maze	Open Field	Light-Dark box
XY-*Sry*	20 (19)	20 (19)	20	20
XX*Sry*	21 (20)	21 (20)	21	21
XX	27	27	27	27
XY-	21	21	21	21
Total	**89 (87)**	**89 (87)**	**89**	**89**

4.2.2 Oestrus cycle

Oestrus status in female subjects was determined by vaginal smearing at the end of each experimental day, following the protocol outlined in Chapter II, 2.2.4.

4.2.3 Anxiety behaviour test battery

All animals were tested in the following order; elevated plus maze, open field, light-dark box, and elevated zero maze. The order of the tests was not pseudorandomised due to considerable practical difficulties. Furthermore, previous literature has suggested that exposure of animals to multiple anxiety tasks does not have a major impact on their behaviour (Lad et al., 2010; McIlwain, Merriweather, Yuva-Paylor, & Paylor, 2001; Paylor, Spencer, Yuva-Paylor, & Pieke-Dahl, 2006). For each mouse, there was a gap of two days between each test. Description of the apparatus and the testing conditions (light intensity etc) can be found in Chapter II, 2.3.1.4-2.3.1.7. Animals were allowed to habituate to the test room for at least 10 minutes prior to testing. Male and female mice were run in a pseudorandom order. For each subject, the number of foecal boli produced was counted, as an additional measurement of anxiogenic behaviour (Henderson, Grazia Turri, DeFries, & Flint, 2004). Between subjects, the apparatus was thoroughly cleaned with 1% acetic acid. Performance was recorded using the Ethovision tracking system (Noldus, U.K.). Ethovision performs calculations of the subjects' movements in a virtual arena with specific zone configurations for each apparatus (see sections below for more details). Variables such as duration, entries in the arms, and rearing were measured in terms of location of the greater body-proportion of subjects. Prior to testing, tracking was calibrated for each apparatus using naïve mice of the same body size and fur colour as the experimental animals. Each session was also recorded using DVD HD recorders (Sony Corp, U. K.) or VCR tapes for further analysis if required.

4.2.3.1 Elevated Plus Maze (EPM)

Subjects were placed in the middle of the maze (Chapter II, 2.3.1.4) and were allowed to explore freely for 5 minutes. For Ethovision tracking purposes, the maze arena was divided into five zones; the two open arm zones, the two closed arm zones, and a middle zone. Data from the two open arm and two closed arm zones were combined, forming two cumulative zones (open and closed). For each of these, the following behavioural measures were collected: frequency of entries, time spent in the zone, distance travelled and average velocity. Additional indices of exploratory and risk-

assessment behaviour were also recorded automatically[4]: stretched attend postures (SAPs, defined as an animal keeping his hind legs in the closed arm, but stretching forward the open arm), head dips (looking over the edge of an open arm) and rearing (an animal standing on its hind feet) (Espejo, 1997b; Rodgers & Cole, 1993). The principal anxiety-related measures were time spent in the open and closed arms, and entries into the open arms; entries into the closed arms and distance travelled were regarded as indices of locomotor activity (Rodgers & Dalvi, 1997). Rarely, animals fell from the open arm of the plus maze during exploration; these mice were excluded from statistical analysis.

4.2.3.2 Elevated Zero Maze

At the beginning of the 5-minute session, animals were placed in a closed compartment, facing the exposed part of the maze (Chapter II, 2.3.1.5). As there was no middle zone the zero maze was comprised of four zones (two open; top/down, and two closed; left/right) and two cumulative zones (open and closed). The behavioural parameters measured were the same as for EPM (see section above). Rarely, animals fell from the open arm of the plus maze during exploration; these mice were excluded from statistical analysis.

4.2.3.3 Open Field Arena (OF)

Mice were placed into a square shaped arena (Chapter II, 2.3.1.6), consistently facing the same wall. Each session lasted for 10 minutes, during which subjects were free to explore. The OF arena was divided into three square areas; a central zone (20x20cm), an intermediate zone (40x40cm) and an outer zone (remaining peripheral region, 60x60cm). The main anxiety-related behavioural measures included the duration of time spent in the centre (the most exposed and therefore considered the most aversive part of the apparatus), the frequency of entries into the central zone, and the latency of first entry into the centre. Distance travelled, and average velocity was recorded as indices of locomotion.

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[4] SAPs and head dips were scored by the experimenter live during each session.

4.2.3.4 Light-dark box

At the beginning of each session, mice were placed in the less aversive dark compartment of the box (see Chapter II, 2.3.1.7) and were free to explore both compartments for 10 minutes. For tracking purposes, the apparatus was divided into two main zones; hidden ('dark' compartment) and white ('light' compartment). The more aversive light zone was further subdivided into two areas; 'near' and 'far' (up to 15cm from partition door, and up to 30cm from the partition door respectively). Data were collected for the following main parameters: duration of time spent in each zone, frequency of entries into each zone, latency of first entry into the light zone, total distance covered, average velocity in the light zone, and rearing in each zone.

4.2.4 Statistical analysis

Statistical analyses were performed using Two Way ANOVA, with SRY DEPENDENCE (i.e. gonadal male or female) and SEX CHROMOSOME COMPLEMENT (XX or XY) as the two main factors. When there was a significant interaction between the two factors, Fisher's Least Significant Difference (LSD) adjustment was used for *post hoc* pairwise comparisons. Data that deviated from normality were transformed using appropriate methods where necessary. When data could not be transformed, Mann-Whitney U-test for non-parametric data was performed. Data are presented as mean values ± standard error of the mean (SEM). P values ≤ .05 were regarded as significant.

4.3 Results

4.3.1 Elevated Plus Maze

As expected, all the mice spent considerably more time in the enclosed areas of the maze, than in the exposed, open arms (219.3±3.9s, 21.5±2s respectively). Within this general effect there was a significant main effect of SRY DEPENDENCE on time spent in the closed arms, whereby gonadally male mice spent less time in these zones than gonadally female mice ($F_{1,83}$=5.269, $p<.05$) (Figure 4.3.1a). There was no main effect of SEX CHROMOSOME COMPLEMENT ($F_{1,83}$= .123, p= .727) nor any SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT interaction ($F_{1,83}$= .420, p= .519) on this measure. No significant effect of SRY DEPENDENCE ($F_{1,83}$= .1564, p= .455), SEX CHROMOSOME COMPLEMENT ($F_{1,83}$=1.002, p= .320) or SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT interaction ($F_{1,83}$=3.015, p= .086) was noted on time spent in the open arms. Further analysis of behaviour on the open arms revealed an interaction in the number of head dips made ($F_{1,83}$=4.949, $p<.05$); XY-*Sry* mice were shown to have made more head dips than XX*Sry* mice (4.7± .8 vs. 1.5± .8 respectively) (p=.057), implying an anxiolytic pattern of behaviour.

Figure 4.3.1a

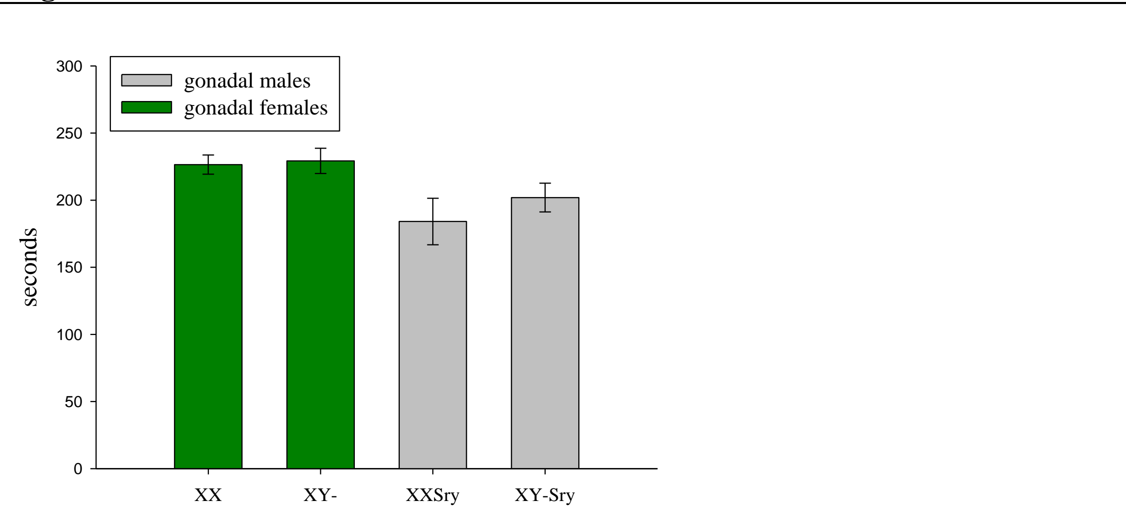

Figure 4.3.1a: Duration of time spent in closed arms of EPM; gonadally male mice, expressing the *Sry* transgene, spent significantly less time than gonadally female mice.

Data from the other measures assessed in the EPM are summarised in Table 4.3.1i below. A significant interaction between SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT was reported on overall average velocity (velocity of open and closed arms combined) ($F_{1,83}$= 4.378, p<.05) whereby gonadally male mice carrying a Y chromosome (XY-*Sry*) were generally faster than gonadally male mice with two XX chromosomes (XX*Sry*) (3.9± .2 cm/s vs. 3.2± .1 cm/s respectively) (p<.05). A main effect of SRY DEPENDENCE was observed in terms of total rears ($F_{1,83}$= 4.424, p<.05); gonadally male mice displayed a significantly greater number of rears than gonadally female mice, denoting greater exploratory and anxiolytic behaviour.

Table 4.3.1i: Summary of additional behavioural measures taken in EPM; descriptive data of the measures are presented in Appendix B, 2.1. Total distance, velocity and rearing refer to measures averaged across closed and open arms. Significant effects are highlighted in bold.

	Behavioural measure	Effect of SRY DEPENDENCE	Effect of SEX CHROMOSOME COMPLEMENT	SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT
Total	Distance travelled in maze (cm)	$F_{1,83}$=1.941, p= .167	$F_{1,83}$=1.803, p= .183	$F_{1,83}$=2.313, p= .132
	Velocity (cm/s)	$F_{1,83}$= .036, p= .850	$F_{1,83}$=1.718, p= .194	**$F_{1,83}$=4.378, p<.05**
	Rearing	**$F_{1,83}$=4.424, p<.05**	$F_{1,83}$= .236, p= .628	$F_{1,83}$= .806, p= .372
Closed arms	Entries	$F_{1,83}$=1.095, p= .298	$F_{1,83}$= .317, p= .575	$F_{1,83}$= .682, p= .411
	Stretch Attends	$F_{1,83}$= .854, p= .358	$F_{1,83}$= .086, p= .769	$F_{1,83}$= .184, p= .669
Open arms	Entries	$F_{1,83}$= .270, p= .605	$F_{1,83}$= .875, p= .352	$F_{1,83}$= 2.118, p= .149
	Head Dips	$F_{1,83}$= .168, p= .683	$F_{1,83}$= .399, p= .529	**$F_{1,83}$= 4.949, p<.05**

4.3.2 Elevated Zero Maze

As in the EPM, overall the mice spent more time in the closed than in the open quadrants of the zero maze (275.4±2.4s vs. 20.1±2.3s, respectively). A significant effect of factor SRY DEPENDENCE was observed on time spent in the open quadrants of the maze ($F_{1,83}$= 3.817, p=.05), with gonadal males spending significantly longer exploring these zones than gonadal females (Figure 4.3.2a, A.). There was no main effect of SEX CHROMOSOME COMPLEMENT ($F_{1,83}$= .212, p= .646) nor any interaction between SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT ($F_{1,83}$= .099, p= .753) on this measure. Therefore, in the zero maze an apparent reduced anxiety phenotype was seen across all the groups possessing *Sry*. There was also a significant effect of SRY DEPENDENCE on frequency of entries into the open quadrants, with gonadally male mice making significantly more entries ($F_{1,83}$= 6.511, p<.05) (Figure 4.3.2a, B.). No significant main effect of SEX CHROMOSOME COMPLEMENT ($F_{1,83}$=2.134, p=.148) nor SRY DEPENDENCE

by SEX CHROMOSOME COMPLEMENT interaction ($F_{1,83}$= .057, p= .813) was noted on open quadrant entries.

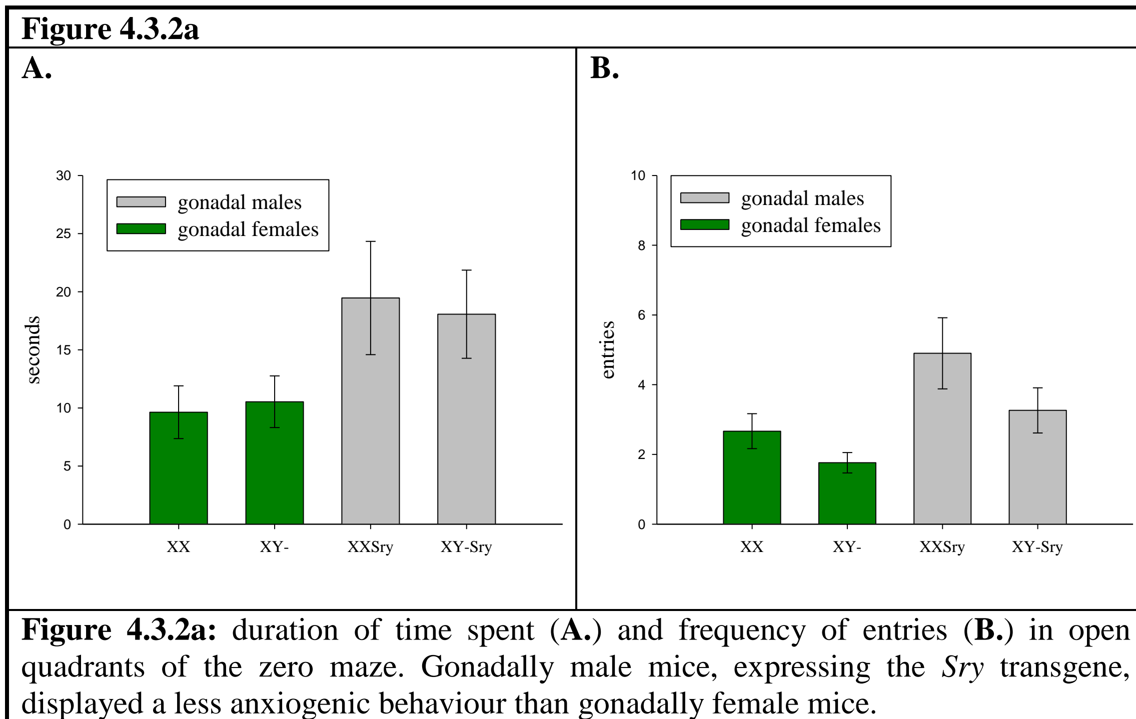

Figure 4.3.2a: duration of time spent (**A.**) and frequency of entries (**B.**) in open quadrants of the zero maze. Gonadally male mice, expressing the *Sry* transgene, displayed a less anxiogenic behaviour than gonadally female mice.

There was a main effect of SRY DEPENDENCE on number of rears (in open quadrants) ($F_{1,83}$=8.016, p<.01) and of stretch attends (in close quadrants) ($F_{1,83}$=11.073, p<.01), with gonadally male mice performing a greater number of both behaviours than gonadally female mice (Figure 4.3.2b). There were no other significant effects of SRY DEPENDENCE, SEX CHROMOSOME COMPLEMENT, or SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT on the secondary measures assayed in the elevated zero maze (Table 4.3.2i).

Table 4.3.2i: Summary of analysis on secondary measures of the open quadrants of the zero maze. Descriptive data of the measures below are presented on Appendix B, 2.2.

	Behavioural measure	Effect of SRY DEPENDENCE	Effect of SEX CHROMOSOME COMPLEMENT	SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT
Open quadrants	Distance (cm)	$F_{1,83}$=2.549, p= .114	$F_{1,83}$= 486, p= .488	$F_{1,83}$= .250, p= .618
	Velocity (cm/s)	$F_{1,83}$= .569, p= .453	$F_{1,83}$= .236, p= .629	$F_{1,83}$= .2685, p= .410
	Head Dips	$F_{1,83}$= .689, p= .409	$F_{1,83}$= .550, p= .460	$F_{1,83}$= .020, p= .888

Figure 4.3.2b

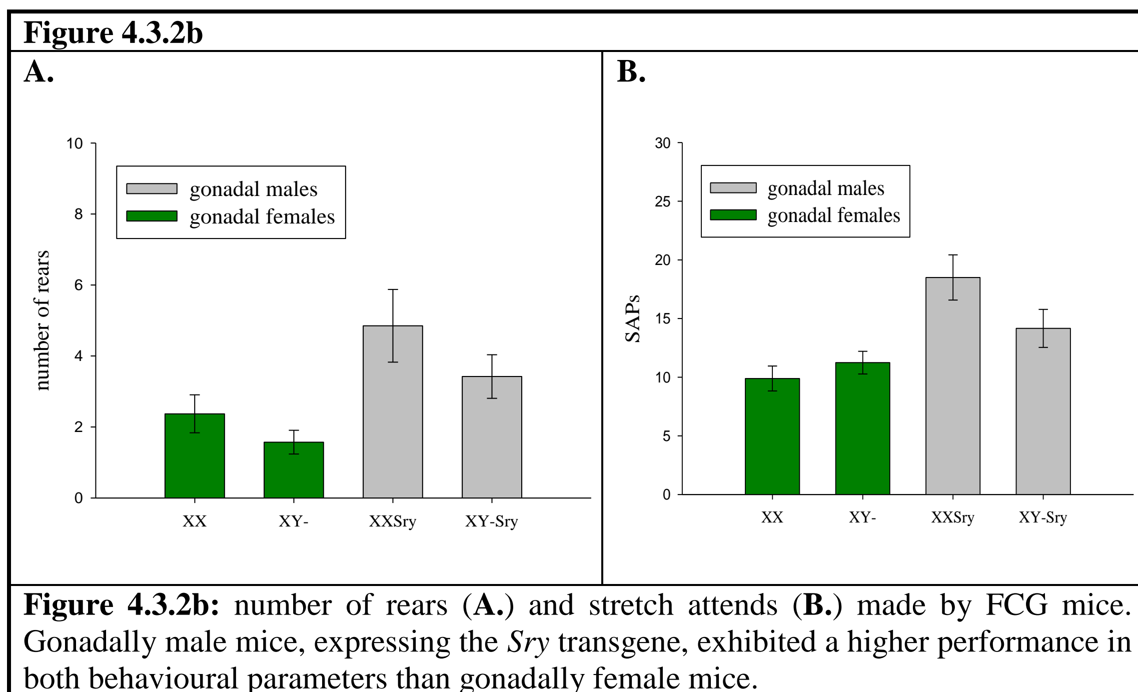

Figure 4.3.2b: number of rears (**A.**) and stretch attends (**B.**) made by FCG mice. Gonadally male mice, expressing the *Sry* transgene, exhibited a higher performance in both behavioural parameters than gonadally female mice.

4.3.3 Open Field

Consistent with being a relatively lower aversive part of the apparatus, all mice spent a greater proportion of their time in the outer zone of the arena, displaying thigmotaxis (circling round the perimeter of the arena keeping close to the outer walls) and avoiding the (presumably) more aversive centre (540.4±4.4s vs. 61.6±4.4s respectively). There were no significant effects on measures indexing anxiety, or exploration in the open field test (Table 4.3.3i). In terms of indices of locomotion, an SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT interaction was

yielded on total distance travelled and total velocity (both measures averaged across the three zones). *Post hoc* LSD comparisons revealed that XX females covered more distance and were faster than XY- (distance: $F_{1,85}$=5.889, p< .05; velocity: $F_{1,85}$=4.388, p< .05).

Table 4.3.3i: Summary of additional behavioural measures taken in OF; descriptive data of the measures are presented in Appendix B, 2.3. Total distance, velocity and rearing refer to measures averaged across all three zones. Significant effects are highlighted in bold.

Zone	Behavioural measure	Effect of SRY DEPENDENCE	Effect of SEX CHROMOSOME COMPLEMENT	SRY DEPENDENCE* SEX CHROMOSOME COMPLEMENT interaction
Total	Distance (cm)	$F_{1,85}$= .003, p= .958	$F_{1,85}$= .298, p= .586	**$F_{1,85}$=5.889, p< .05**
	Velocity (cm/s)	$F_{1,85}$= .032, p= .858	$F_{1,85}$=1.491, p= .225	**$F_{1,85}$=4.388, p< .05**
	Rearing	$F_{1,85}$=.676, p= .413	$F_{1,85}$= .008, p= .928	$F_{1,85}$=3.306, p= .073
Outer	Frequency	$F_{1,85}$=.961, p= .330	$F_{1,85}$= .067, p= .797	$F_{1,85}$=2.989, p= .087
	Duration	$F_{1,85}$=.416, p= .521	$F_{1,85}$= .730, p= .395	$F_{1,85}$=2.199, p= .142
Intermediate	Frequency	$F_{1,85}$=1.107, p= .296	$F_{1,85}$= .024, p= .877	$F_{1,85}$=3.104, p= .082
	Duration	$F_{1,85}$=.204, p= .653	$F_{1,85}$= .263, p= .610	$F_{1,85}$=1.783, p= .185
Central	Frequency	$F_{1,85}$=.194, p= .660	$F_{1,85}$= .077, p= .782	$F_{1,85}$=3.599, p= .061
	Duration	$F_{1,85}$=.406, p= .526	$F_{1,85}$= .657, p= .420	$F_{1,85}$=1.968, p= .164

4.3.4 Light-Dark box

Again as expected, animals generally spent more time in the dark compartment of the box than in the bright, aversive section (520.3±5.2s vs. 61.6±3.9s respectively). There was a significant effect of SEX CHROMOSOME COMPLEMENT on duration of time spent in the less aversive dark compartment ($F_{1,85}$=5.124, $p<.05$), with karyotypically female mice (XX) spending less time in this zone than karyotypically male mice (XY) (Figure 4.3.4a; A.). There was no significant effect of SRY DEPENDENCE ($F_{1,85}$= .003, p= .959) nor any significant interaction between SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT on this measure ($F_{1,85}$=3.383, p=.069). A significant effect of SEX CHROMOSOME COMPLEMENT was also evident on behaviour in the light box, whereby mice possessing two X chromosomes spent more time in the near zone (zone up to 15cm from partition door) than mice with a male XY karyotype ($F_{1,85}$=9.438, $p<.01$) (Figure 4.3.4a; B.), thus displaying a more anxiolytic pattern of behaviour (consistent with spending less time in the dark box) than XY carriers. There was no main effect of SRY DEPENDENCE or SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT on this measure ($F_{1,85}$= .0017, p= .898, and $F_{1,85}$= .567, p= .454, respectively). Moreover, a significant SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT interaction was noted on duration of time spent in the far end of the light box (far zone up to 30cm from partition door) ($F_{1,85}$=4.163, $p< .05$); *Post hoc* LSD comparisons revealed that XX females spent more time in the most aversive area than XY- (p= .05). There was no main effect of SRY DEPENDENCE or SEX CHROMOSOME COMPLEMENT on this measure ($F_{1,85}$= .042, p= .838, and $F_{1,85}$= .403, p= .527, respectively).

Figure 4.3.4a

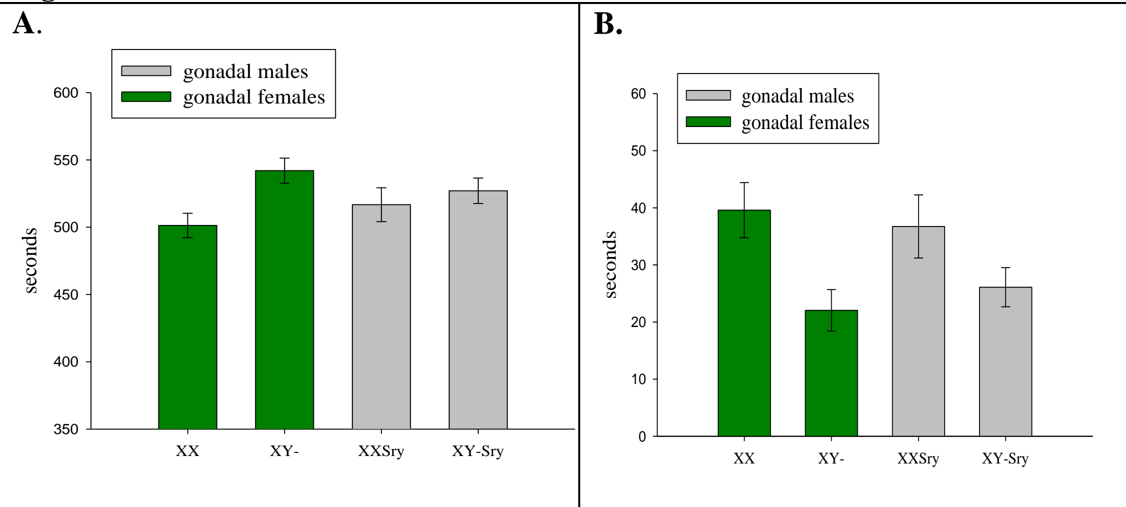

Figure 4.3.4a. Mice with XX karyotype spent less time in the dark zone (**A.**) and more time in the near part (up to 15cm from partition door) of the bright area (**B.**) of the light-dark box than mice with XY karyotype.

Figure 4.3.4b

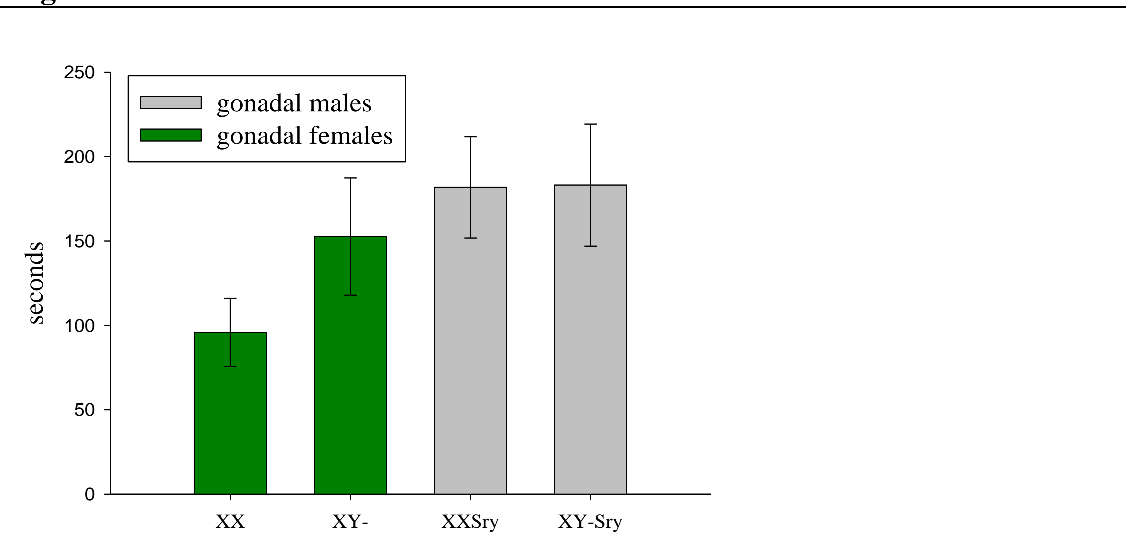

Figure 4.3.4b: Latency of first entry into the light compartment of the box. Gonadally female mice made their first entry into the light compartment quicker than gonadally male mice.

We noted a significant effect of SRY DEPENDENCE on latency of first entry into the light compartment, whereby gonadally female mice had reduced latencies compared to gonadally male mice ($F_{1,85}$=4.963, p<.05) (Figure 4.3.4b). No effects of SEX CHROMOSOME COMPLEMENT, nor any interaction between SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT were evident on this parameter ($F_{1,85}$= .136, p= .714 and $F_{1,85}$= .398, p= .530 respectively). We did not

observe any further significant effects on indices of exploration or activity assayed in this test (Table 4.3.4i).

Table 4.3.4i: Summary of additional findings of light-dark box analysis; descriptive data of the measures below are presented in Appendix B, 2.4. Total distance, velocity and rearing refer to measures averaged across near and far zones of the light compartment.

Zone	Behavioural measure	Effect of SRY DEPENDENCE	Effect of SEX CHROMOSOME COMPLEMENT	SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT
Total	Distance (cm)	$F_{1,85}$=2.051, p= .156	$F_{1,85}$= .377, p= .541	$F_{1,85}$= .362, p= .549
	Velocity (cm/s)	$F_{1,85}$= .895, p= .347	$F_{1,85}$= 1.490, p= .226	$F_{1,85}$= .541, p= .464
	Rearing	$F_{1,85}$= .061, p= .805	$F_{1,85}$= 2.072, p= .154	$F_{1,85}$= 2.116, p= .149
Dark	Frequency	$F_{1,85}$= .004, p= .952	$F_{1,85}$= .266, p= .607	$F_{1,85}$=3.889, p= .052
Near	Frequency	$F_{1,85}$= .002, p= .964	$F_{1,85}$=1.955, p= .166	$F_{1,85}$=2.161, p= .145
Far	Frequency	$F_{1,85}$= .098, p= .755	$F_{1,85}$= .188, p= .666	$F_{1,85}$=3.170, p= .079
	Latency	$F_{1,85}$= .147, p= .703	$F_{1,85}$= .111, p= .740	$F_{1,85}$= .577, p= .450

4.3.5 Foecal boli and stage of oestrus

For each behavioural task, the number of foecal boli were measured; using this well established index of anxiety (Flint et al., 1995; Willis-Owen & Flint, 2006) the analysis did not reveal any significant differences between the different genotypes (Table 4.3.5i). In tasks where a significant main effect of SRY DEPENDENCE was observed, Analysis of Covariance (ANCOVA) was performed, with GENOTYPE (XX and XY-) as the fixed factor and OESTROUS STAGE as the covariate, in order to test for oestrous-stage related effects. In no case did this analysis yield any significant effect of OESTROUS STAGE (see Appendix B, 2.6-2.8). In other words, the differing hormonal levels of female mice at different stages of oestrus did not account for any significant genotype effects observed.

Table 4.3.5i. Analysis of defecation among the FCG mice, on anxiety-related tasks. Descriptive data are presented on Appendix B, 2.5

Behavioural task	Effect of SRY DEPENDENCE	Effect of SEX CHROMOSOME COMPLEMENT	Effect of SRY DEPENDENCE*SEX CHROMOSOME COMPLEMENT interaction
Elevated Plus Maze	$F_{1,83}$= .515, p= .475	$F_{1,83}$= .102, p= .750	$F_{1,83}$= .302, p= .584
Zero Maze	$F_{1,82}$= .000, p= .987	$F_{1,82}$=1.281, p= .261	$F_{1,82}$= .278, p= .599
Open Field	$F_{1,85}$= .032, p= .858	$F_{1,85}$= .095, p= .759	$F_{1,85}$= .001, p= .978
Light-Dark box	$F_{1,85}$= .194, p= .660	$F_{1,85}$= .020, p= .888	$F_{1,85}$= .140, p= .709

4.3.6 Summary data for factors **SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT assaying anxiety-related behaviours in the FCG model**

Table 4.3.6i summarises the significant main effects and interaction with factors SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT across the multiple assays for anxiety-related behaviours in the FCG model. Briefly, gonadally male mice (XY-*Sry* and XX*Sry*) displayed a more anxiolytic behaviour than gonadally female mice (XX and XY-) in the elevated plus maze and zero maze. Furthermore, XY-*Sry* mice were faster (average velocity) and performed more head dips in the open arms of the elevated plus maze than XX*Sry* mice. Performance on the open field and light-dark box revealed a different pattern of behaviour. In terms of indices of locomotion in the open field, XX mice covered greater distance across the arena and were faster than XY- mice. In the light-dark box, XX carriers (XX and XX*Sry*) spent less time in the dark, least aversive zone, and more time in the near bright zone than XY carriers (XY-*Sry* and XY-), a behavioural pattern consistent with less anxiety. Furthermore, XX female mice spent more time in the far bright zone than XY- female mice. Finally, it should be noted that a *Sry*-dependent effect was observed in terms of latency to enter the bright area of the box, as gonadally female mice made the first entrance earlier than gonadally male mice, which could arguably be regarded as an index of anxiolysis.

Table 4.3.6i. Summary of significant effects in the FCG model on behaviour in anxiety-related tasks

Effects	Behavioural measures
Significant effects of SRY DEPENDENCE	• EPM - Duration in closed arms (↓ in gonadal males) - Total rearing (↑ in gonadal males) • Zero Maze - Frequency of entries in open quadrants (↑ in gonadal males) - Duration in open quadrants (↑ in gonadal males) - Total rearing (↑ in gonadal males) - Stretch attend postures (↑ in gonadal males) • Light-Dark box - Latency to enter bright area (↑ in gonadal males)
Significant effects of SEX CHROMOSOME COMPLEMENT	• Light-Dark box - Duration in dark area (↑ in XY karyotype) - Duration in the near area (↓ in XY karyotype)
Significant interaction between SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT	• EPM - Average velocity (fastest in XY-*Sry*) - Head dips in the open arms (most in XY-*Sry*) • Open Field - Average velocity (fastest in XX) - Average distance across all zones (greatest in XX) • Light-Dark box - Duration in the far area (greatest in XX)

4.3.7 Additional comparisons with XY wildtype mice

Wildtype MF1 XY mice were run alongside the FCG mice during behavioural testing. This was done to enable a further “probe” analysis to determine the extent to which XY males were behaviourally equivalent to XY-*Sry* males, and to test whether effects of SRY DEPENDENCE or SEX CHROMOSOME COMPLEMENT on behaviour manifest in the FCG cross had any relevance to sex differences in normal, wildtype animals. *A priori*, three experimental groups were compared (XY, XY-*Sry* and XX; see footnote 5 below)[5], using One Way ANOVA with GENOTYPE as the independent factor; *post hoc* comparisons were performed using Bonferroni post-hoc test; this *post hoc* option was chosen, as Bonferroni is a conservative test, which controls for Type I error well. These additional analyses were undertaken for any measures that had been shown to be sensitive to either SRY DEPENDENCE or SEX CHROMOSOME COMPLEMENT in the main analysis of the FCG mice, as those measures would probably be the most informative in terms of sexual dimorphisms in normal mice.

4.3.7.1 Elevated Plus Maze

The three experimental groups were compared on the behavioural parameters of duration of time spent in closed arms and total rearing. No significant differences were observed in either case ($F_{2, 68}$=2.449, p= .09; $F_{2, 68}$=2.366, p= .1 respectively; Figure 4.3.7.1a, and b), suggesting that all three groups displayed similar anxiety-related behaviour in the task.

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[5] These groups were chosen as they represented the best candidates for our “probe” analysis. XY and XX mice were normal male and female animals respectively, while XY-*Sry* males differed to XY males in that the XY-*Sry* males expressed the transgenic version of *Sry* and the XY males endogenous *Sry*.

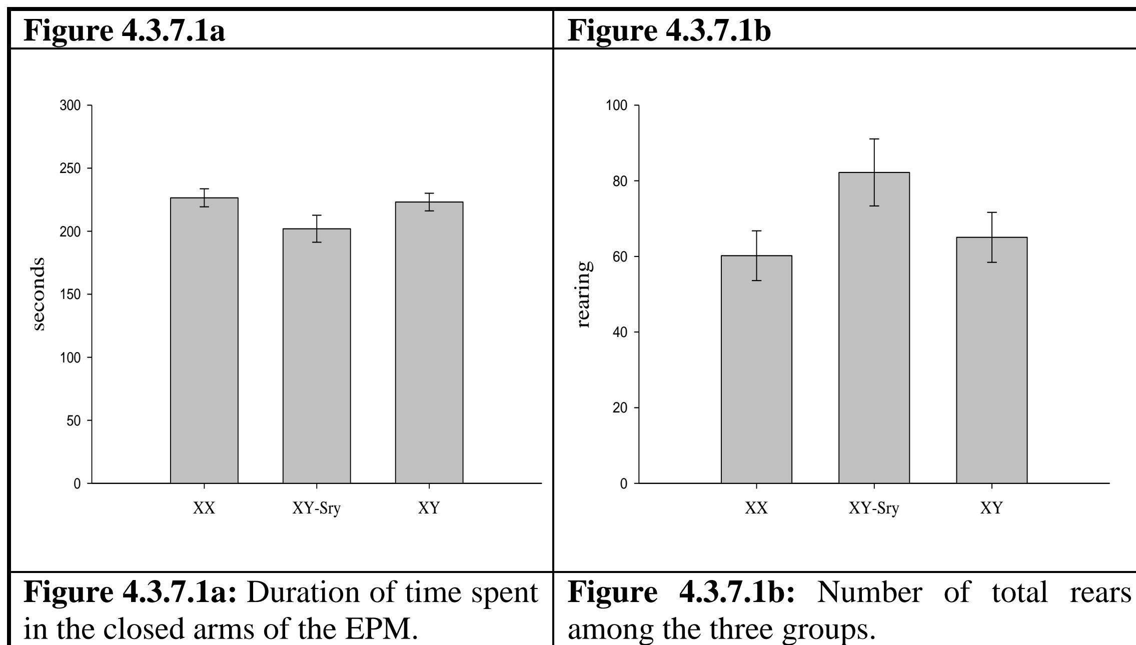

Figure 4.3.7.1a: Duration of time spent in the closed arms of the EPM.

Figure 4.3.7.1b: Number of total rears among the three groups.

4.3.7.2 Elevated Zero Maze

Comparison of the main three groups yielded a significant main effect of GENOTYPE on duration of time spent in open quadrants of the zero maze ($F_{2,68}$=6.129, p<.01), a sensitive index of anxiety. *Post hoc* comparisons showed that XX mice displayed a more anxiogenic pattern of behaviour than XY mice (p<.05), as they spent less time in the open quadrants of the maze than wildtype male mice. Importantly, there were no significant differences in behaviour between XY and XY-*Sry* male mice (Figure 4.3.7.2a, A.), suggesting that the presence of *Sry* transgene did not have major impact on this specific behaviour. One-way ANOVA did not reveal any significant differences on frequency of entries into the open quadrants ($F_{2, 68}$=1.841, p= .167) (Figure 4.3.7.2a, B). A significant main effect of GENOTYPE was also noted on total number of rears (in open quadrants; $F_{2, 68}$=3.556, p< .05). *Post hoc* comparisons showed that XX mice performed less rears than XY mice (p<.05), consistent with the anxiogenic pattern displayed on duration of time spent in the open quadrants (Figure 4.3.7.2b, A.). Analysis of stretch attend postures in the closed quadrants (SAPs) also showed a significant effect of GENOTYPE ($F_{2, 68}$=3.510, p<.05); although XX mice showed a trend towards fewer SAPs than both gonadal male groups, *post hoc* comparisons were not significant (Figure 4.3.7.2b, B.).

Figure 4.3.7.2a

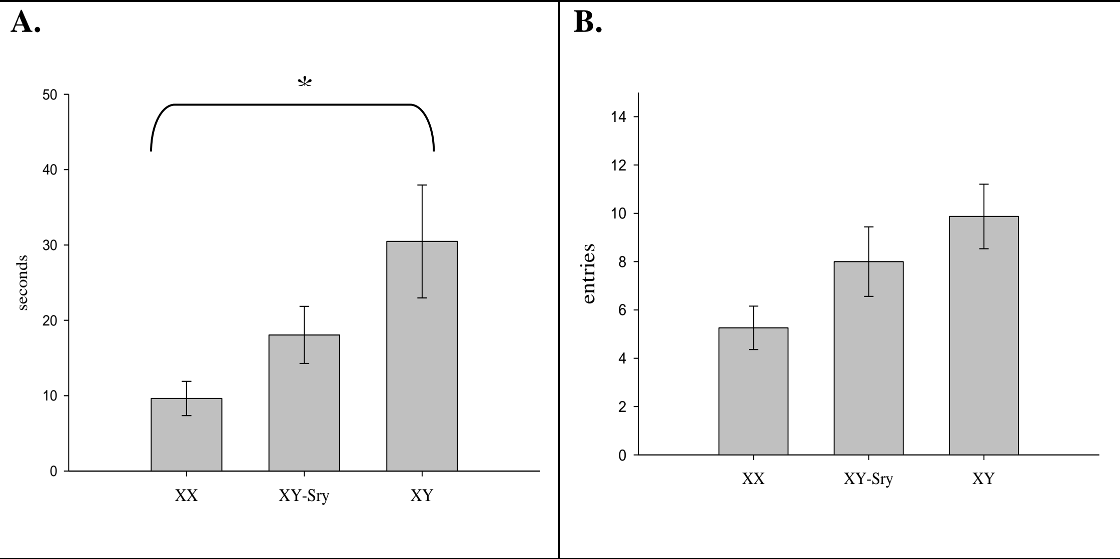

Figure 4.3.7.2a:A. Duration of time spent in open areas of zero maze. XX mice differed from XY mice. **B.** Frequency of entries in the open areas of zero maze. *p<.05.

Figure 4.3.7.2b

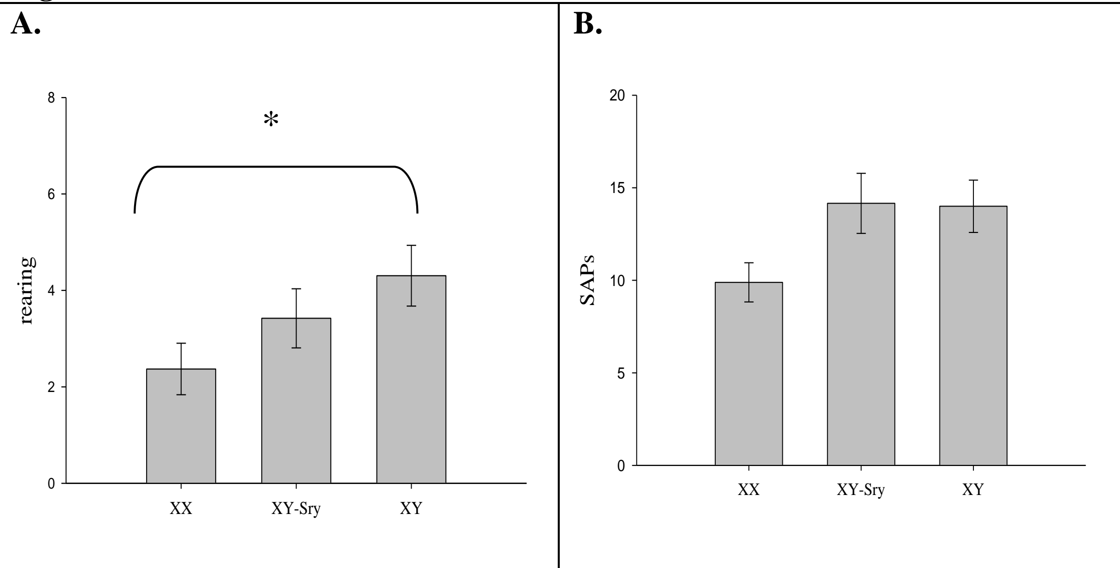

Figure 4.3.7.2b: A. Number of total rears made by the three groups. XX mice differed from XY mice **B.** Number of stretch attends exhibited by the three groups. *p<.05.

4.3.7.3 Light-Dark box

One-way ANOVA did not reveal any significant differences among the three groups on duration of time spent in the dark compartment (effect of GENOTYPE, $F_{2,69}$=2.716, p=0.07, Figure 4.3.7.3a; A.). Similarly, there was no significant effect of GENOTYPE on time spent in the near area of the light box ($F_{2,69}$=2.555, p= .08); (Figure 4.3.7.3a; B.) or on latency to make the first entry into the bright compartment ($F_{2,69}$=2.908, p= .062) (Figure 4.3.7.3b).

Figure 4.3.7.3a

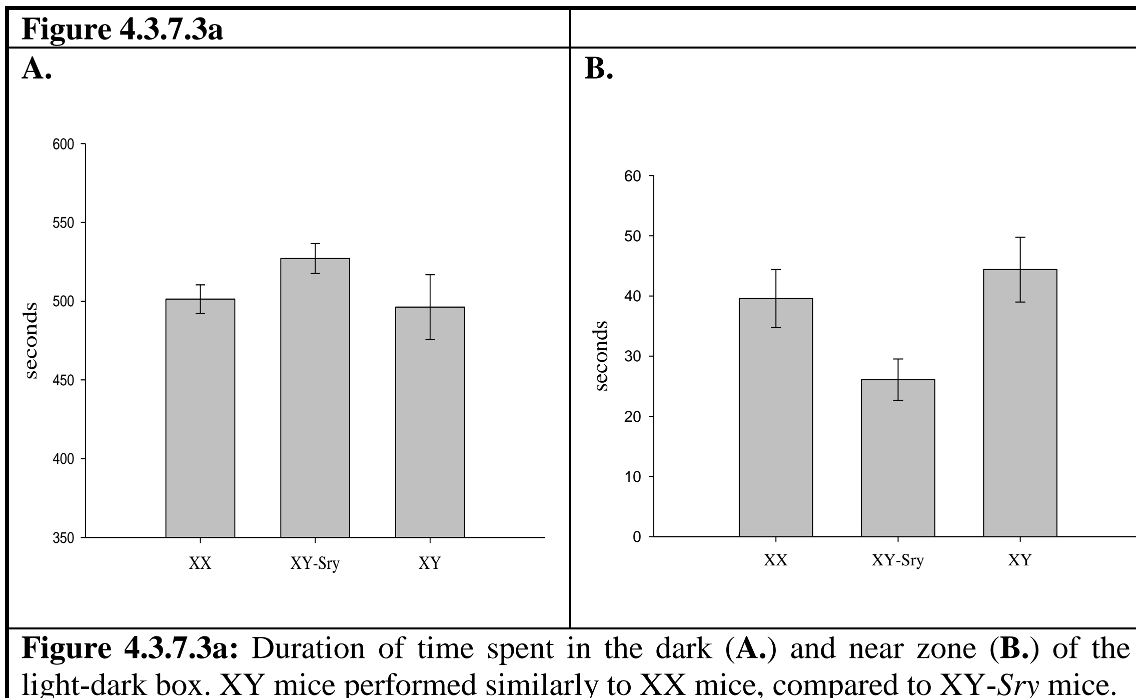

Figure 4.3.7.3a: Duration of time spent in the dark (**A.**) and near zone (**B.**) of the light-dark box. XY mice performed similarly to XX mice, compared to XY-*Sry* mice.

Figure 4.3.7.3b

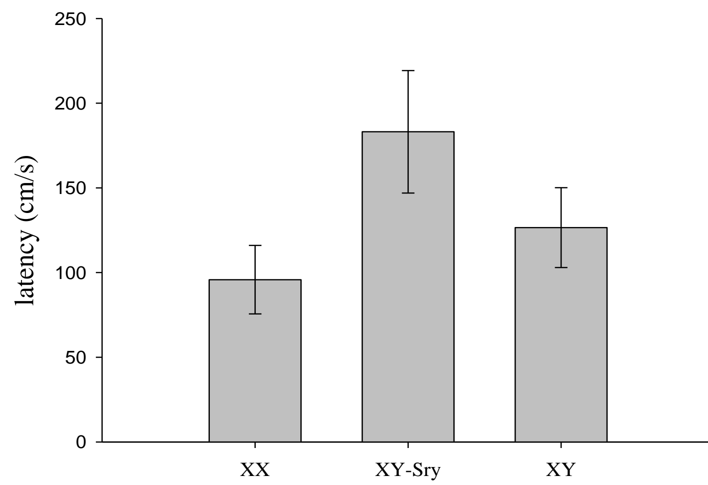

Figure 4.3.7.3b: Latency to make the first entry into the bright compartment of the light-dark box.

4.4 Discussion

The aim of this chapter was twofold: i) to examine whether *Sry* and/or sex chromosome complement had an effect on anxiety-related behaviours in four well-characterised paradigms and ii) to test whether reliable behavioural effects arising from *Sry* or other sex-linked genetic mechanisms may explain sex differences in anxiety-related behaviours in normal mice.

In this study care was taken to control for potential confounds, which could have influenced the data, such as hormonal state (oestrous cycle) of female mice, as well as possible odour effects arisen by testing male and female mice in the same apparatus. With regards to the former issue, vaginal smearing was performed daily during the testing period and oestrous stage was included as a covariate on measures where there was a SRY DEPENDENCE effect; this analysis showed no significant effects of this index of female hormone status on the anxiety-related measures assayed here. This null finding is in contrast with some studies, which have implied a role of female hormones, notably oestrogen, on anxiety and fear-related behaviours (Toufexis, et al., 2006). Notwithstanding, there have been inconsistencies among studies about the specific role of oestrogen on tasks tapping anxiety (anxiogenic or anxiolytic) (Morgan & Pfaff, 2001), which in combination with other factors, such as implementation of different methodological designs (using cycling female animals or gonadectomised females, which have been subsequently given oestrogen supplements) and the use of different species (rats vs mice) and strains, create a rather complicated picture. Odour effects were controlled by thoroughly cleaning the apparatus between each session (1% acetic acid). Another important issue regarding the methodological design is the lack of pseudo-randomisation of the anxiety tasks, which could have led to 'order effect' confounds. As already mentioned in section 4.2.3 all animals underwent testing in the same order mainly due to practical difficulties. Furthermore, according to previous literature, subjects can be tested in multiple tests of anxiety in the same order without impact on performance (Lad, et al., 2010; McIlwain, et al., 2001; Paylor, et al., 2006). It is thus unlikely that any order effects would have altered the data. A final issue that should be addressed concerns the secondary probe analysis conducted between XY-*Sry*, XY, and XX mice. We opted in this study not to analyse all five genotypes (FCG mice and XY wildtype mice) simultaneously as the aim was to address two different hypotheses. The main

analysis focused on whether there were any *Sry*-dependent and/or sex chromosome complement-dependent effects on anxiety-related behaviour (FCG model). The probe analysis, on the other hand, investigated to what extent (if at all) XY-*Sry* mice differ from XY wildtype mice and whether normal male and female mice (XY and XX) differed in aspects of anxiety-related behaviour. For this latter analysis, *post hoc* comparisons were performed using the conservative Bonferroni correction, in an attempt to minimise potential inflation of Type I error.

A main finding from this chapter was an influence of SRY DEPENDENCE on the measures of anxiety in the conceptually similar elevated plus and zero maze tasks; specifically, in the zero maze, gonadally male mice possessing a *Sry* transgene (both XX*Sry* and XY-*Sry*) spent longer exploring the open, more aversive, areas of the apparatus than gonadally female mice (and less time exploring the less aversive enclosed areas); more subtle effects were noted in the EPM, where gonadally male mice spent less time in the closed arms of the apparatus than gonadally female mice. The overlapping findings on elevated plus and zero mazes, which were performed at either end of the behavioural testing regime, imply that undergoing the intervening tests (open field and light-dark box) had little effect on subsequent behavioural performance in the mice. Furthermore, since gonadal males and females exhibited equal within-test activity on the mazes, as indexed by distance travelled and velocity, it is likely that time spent in the various arms of the mazes reflects anxiety-related behaviour *per se* rather than a consequence of mundane effects on activity levels. Additionally, if the pattern of behaviour in the tests of anxiety was linked to activity in some way, one might expect the increased exploration of the aversive parts of the mazes to be associated with increased activity in general. However, this is not the case, as in tasks assessing activity independently of the mazes (e.g. homecage paradigm and locomotor activity chambers) it was the gonadal females that were the more active (Chapter III, sections 3.3.2.1 and 3.3.2.2). Also consistent with the idea that the presence of *Sry* may, under some circumstances, be anxiolytic, gonadally male mice engaged in more exploratory behaviours (rearing and stretched-attend postures), particularly in the elevated zero maze (Shepherd, et al., 1994), than gonadally female mice. Importantly, these findings implicating *Sry* in anxiety to an extent replicate and extend those previously observed in the laboratory using the FCG model (P. M. Y. Lynn, 2010).

A second main finding of the chapter was that SEX CHROMOSOME COMPLEMENT could influence anxiety-related behavioural measures in the light-dark box paradigm. Specifically, possessing a female karyotype rather than a male one caused mice to spend a greater proportion of their time exploring the more aversive light box. This apparently contradictory result, together with the dissociation between *Sry*-dependent/sex chromosome effects on some tests but not others, focuses attention on the likelihood that the four different tasks, whilst having some degree of overlap, do in fact assay distinct aspects of anxiety, in particular with regard to the distinction between the two maze tasks and the Open Field, Light-Dark box assays; there is evidence from behavioural, pharmacological and genetic studies that this may indeed be the case (Belzung & Le Pape, 1994; Beuzen & Belzung, 1995; Griebel, Belzung, Perrault, & Sanger, 2000; Prut & Belzung, 2003; Simon, et al., 1994). In this light, it would appear that both *Sry* (effect of SRY DEPENDENCE) and other sex chromosome linked genes (effect of SEX CHROMOSOME COMPLEMENT) can influence these dissociable components of anxiety.

Our behavioural findings using the FCG model contrast with those of McPhie-Lalmansingh et al. (2008) who reported no effects of SRY DEPENDENCE or of SEX CHROMOSOME COMPLEMENT on anxiety-related measures in the elevated plus maze and open field. One possible reason for this discrepancy is the different background strains used (C57BL/6J(McPhie-Lalmansingh, et al., 2008), MF1 here); inter-strain differences in anxiety parameters have been documented in several studies (An, et al., 2011; Clement, Calatayud, & Belzung, 2002; Griebel, et al., 2000) and it could, therefore, be valid to argue for the existence of different characteristics, relevant to anxiety-related behaviour, between those two strains[6]. There were also inter-laboratory differences in the protocol for the anxiety tests, and it is well known that assays of emotional functioning are especially sensitive to such procedural details (Lad, et al., 2010; Whalsten, et al., 2006). Perhaps most importantly, we did not gonadectomise the mice (to avoid causing potential stress and discomfort, as well as due to the fact that we were interested in organisational and activational effects of gonadal hormones) and housed them in same-sex groups throughout testing. By opting for non-gonadectomy and group housing, we attempted to minimise stress generated by these factors, which could potentially affect subjects' performance in the

[6] MF1 and C57BL/6J have been shown to differ in locomotor activity (LMA) sensitivity to MK-801, a NMDA receptor antagonist (Kalinichev, Bate, Coggon, and Jones, 2007).

tasks. In contrast, in the study by McPhie-Lalmansingh and colleagues, mice were gonadectomised prior to testing and singly-housed thereafter.

As noted above, it is clear from our present findings that *Sry* and sex chromosome complement could both play roles in aspects of anxiety-related behaviour, albeit in a somewhat complex manner. Detailed speculation about the biological mechanisms underlying these complex effects will require more data but in terms of *Sry*-dependent effects it is plausible that *Sry* could influence anxiety-related behaviours such as those seen in the elevated plus and zero mazes in a cell autonomous manner, independently of testosterone secretion e.g. via influences on monoaminergic systems. Specifically, dopaminergic receptors have been implicated in murine performance in the elevated plus maze, and drugs targeting D2 receptors have been shown to exert an anxiolytic effect (De la Mora, Gallegos-Cari, & Arizmendi-Garcia, 2010; Rodgers, Nikulina, & Cole, 1994; Simon, Panissaud, & Costentin, 1993; Wall, Blanchard, Yang, & Blanchard, 2003). Alternatively, *Sry*-dependent effects on behaviour may be mediated by gonadal hormone secretions, notably testosterone; there is strong evidence that testosterone levels may influence anxiety-related behaviours in the elevated plus maze in rodents (Aikey, Nyby, Anmuth, & James, 2002; Edinger & Frye, 2005; Frye & Seliga, 2001; Osborne, et al., 2009). Our behavioural analyses here do not readily allow for dissociation between direct and indirect (hormonally-mediated) effects of *Sry*; this issue will be further addressed in Chapter V.

The sex chromosome effects seen in the Light-Dark box could also be mediated in several ways; by expression of Y-linked genes only in XY mice, altered X-linked gene dosage in XX and XY mice/hemizygosity for X-linked loci in XY mice, or the differential expression of X-linked imprinted genes in XX and XY mice (Davies & Wilkinson, 2006; Kopsida, et al., 2009).In mice, sex-linked chromosomal regions have been postulated to influence open field and light-dark box behaviours. Specifically, quantitative trait loci (QTL)[7] on the X chromosome have been shown to potentially play a role in some parameters of open field and light-dark box, notably activity in novel environments and defecation (Henderson, et al., 2004; Willis-Owen & Flint, 2006).

[7] QTL constitute DNA regions containing genes implicated in a quantitative trait (in this case anxiety-related behaviours).

The work in this chapter also highlighted some significant interactions between SRY DEPENDENCE and SEX CHROMOSOME COMPLEMENT, although these were ancillary measures of anxiety-related behaviour. Potentially, these could be explained by molecular interactions between *Sry*-dependent pathways and pathways affected by sex-linked genes other than *Sry*. For example, the androgen receptor (AR) gene is X-linked and its protein product mediates the intracellular effects of testosterone (Avila, Zoppi, & McPhaul, 2001; Hiipakka & Liao, 1998; Mohlig et al., 2011).

Studies using the FCG mice may provide insights into the molecular and physiological basis of sex differences in behaviour in wildtype male and female mice. In our probe analysis XX mice were compared with XY wildtype and XY-*Sry* mice. XY-*Sry* differ to XY wildtype on *Sry* expression, as they possess a *Sry*-transgene, which is located on an autosomal chromosome (and not on the Y) and has higher expression levels to the endogenous gene (P. M. Y. Lynn, 2010). There were no significant pairwise comparisons between XY-*Sry* and XY mice on the behavioural measures examined in this Chapter, suggesting that the *Sry* transgene did not affect male subjects' performance. Perhaps the most interesting finding from the FCG experiment was a *Sry*-dependent effect on open quadrant time in the elevated zero maze. Here, we showed that wildtype males (and XY-*Sry* males) spend a greater proportion of their time exploring the open quadrants of the elevated zero maze than do wildtype females, a finding supported by some (An, et al., 2011; Chiba, et al., 2009) but not all studies (Gioiosa, et al., 2007; Voikar, et al., 2001). This discrepancy could be attributed to many factors, such as strain differences, and inter-laboratory protocols. Importantly, when taken together, these data indicate that the sex difference seen in wildtype mice in behaviour on the elevated zero maze is likely to be due to *Sry*-dependent effects (direct or indirect) rather than to sex chromosome effects.

4.4.1 Summary points

- In the FCG model expression of the *Sry* transgene (XY-*Sry*, XX*Sry*) was associated with behaviours that could be interpreted as showing reduced anxiety; relatively increased time on the exposed arms of the zero maze and other exploratory/risk assessment behaviours, such as stretch attends, head dips (and relatively decreased time in the non-aversive closed arms of the

EPM); the behavioural data do not distinguish between direct and indirect *Sry*-dependent effects.

- The data from the light-dark box test of anxiety did not follow the same pattern of '*Sry*-dependent anxiolysis'. Instead, here there was a main effect of sex chromosome complement consistent with the possibility that sex-linked genes other than *Sry* can also influence anxiety related behaviours; the data do not allow any conclusions as to the precise gene mechanisms underlying these main effects.
- Dissociations between *Sry*-dependent and sex chromosome effects across the behavioural tasks are likely to result from individual tasks assaying different aspects of anxiety.
- In terms of the key issue of the physiological relevance of the data obtained with the FCG model, XY-*Sry* males behaved equivalently to wildtype XY males on all measures assessed, and, on average, both groups spent a longer time exploring the open quadrants of the zero maze than wildtype females. Hence, sex differences in wildtype mice on this main index of anxiety are likely to be due to effects of the endogenous *Sry* rather than sex chromosome effects.

Chapter V

Further analysis of Sry-dependent effects on behaviour in the Four Core Genotype (FCG) model and wildtype XY mice; correlation with serum testosterone levels and brain expressed Sry

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5.1 Introduction

A main finding stemming from the analysis of FCG mice in Chapter IV was a potential role for *Sry* on anxiety-related behaviour. Specifically, gonadally male mice possessing the *Sry* gene (XY-*Sry*, XX*Sry*), under some circumstances displayed a reduced tendency towards anxiety-related behaviours than gonadally female mice (XY-, XX). Consistent with the idea that the presence of the *Sry* gene may somehow influence anxiety, wildtype male mice (XY) were shown to behave similarly to their transgenic *Sry* counterparts, displaying, in general, less anxiety-related behaviour than wild type female mice (XX). In previous work in the laboratory (P. M. Y. Lynn, 2010) we obtained findings consistent with other *Sry*-dependent effects on behaviour, namely apparent enhanced learning in a maze-based two-way visual discrimination test, whereby gonadally male mice carrying the *Sry* gene (XY-*Sry*, and XX*Sry*) made fewer acquisition errors than gonadally female mice lacking the *Sry* gene (XX and XY-); this previous study also revealed *Sry*-dependent effects on the latency to collect the reinforcer across the acquisition phase (gonadal males collected more rapidly than gonadal females), but not on the speed of commencing a trial, or on the latency to make a choice (P. M. Y. Lynn, 2010).

Theoretically, the above *Sry*-dependent effects on behaviour could be attributed to either a direct, cell autonomous effect of brain expressed *Sry* or to an indirect, testosterone-mediated, effect of the gene. In an attempt to begin to dissociate between these possibilities, the first experiment in this chapter (Experiment 1) examined *Sry* expression in brain and serum testosterone levels in gonadally male mice from the FCG which had previously been behaviourally defined in the work

reported in Chapter IV, and then correlated these physiological measures with the behavioural measures of anxiety which we had previously shown to be *Sry*-dependent (Chapter IV). In parallel, we also compared brain *Sry* expression/serum testosterone levels in a small number of wild type XY males to determine the extent to which the physiology of gonadally male mice from the FCG cross resembled that of genetically unmodified males. This was especially important with regard to levels of *Sry* expression in brain tissue as previous work has shown that the transgenic (ectopic) expression of *Sry* transgene in brain is much higher than endogenous expression from the Y chromosome in wild type male mice (P. M. Y. Lynn, 2010).

We further addressed the important issue of how physiologically relevant the Sry-dependent effects obtained in the FCG model were to normal male behaviour in a second experiment (Experiment 2) by explicitly testing an additional (i.e. not the animals from Chapter IV) large cohort of XY wildtype male mice on the assays which had given rise to Sry-dependent effects in the FCG model (i.e. the previously mentioned tests of anxiety and learning) and then determining the extent to which any within-group variability in behaviour correlated with *Sry* brain expression or serum testosterone levels. We hypothesised, tentatively, based upon previous data, that wildtype XY male mice with the highest levels of brain *Sry* expression and/or serum testosterone would exhibit the fewest anxiety-related behaviours and would learn the visual discrimination with the fewest errors.

5.2 Materials and Methods

Experiment 1

5.2.1 Subjects and animal husbandry

Since this chapter is a continuation of the study described in Chapter IV, the same FCG mice were used and the same procedures were implemented (see Chapter IV, section 4.2.1; n=112: XX=27, XY-*Sry* =21, XX*Sry*=21, XY-*Sry*=20, XY=23). A subset of male mice (youngest mice) from the FCG cross was used for assaying brain *Sry* expression (n=20) and serum testosterone analysis (n=21). Additionally, *Sry* expression and testosterone levels were measured in age-matched wildtype XY male MF1 mice (n=12 and n=14 respectively; same wildtype XY mice used for behavioural analysis in Chapter IV).

5.2.2 Culling protocol

Upon completion of the behavioural testing reported in Chapter IV, animals were culled using the protocol outlined in Chapter II, section 2.4.

5.2.3 Brain Sry expression; RNA extraction and cDNA synthesis

Thirty two gonadally male mice (12 XY-*Sry*, 8 XX*Sry*, and 12 XY) were sacrificed by cervical dislocation and hemi-brain tissue was dissected on ice (see Chapter II, section 2.7). RNA was extracted from homogenised tissue (at 4ºC in a FastPrep FP120 micro-homogenizer; MB Biomedicals, U.S.) in accordance with standard protocols (Chapter II, section 2.8.1). RNA quality and concentration were assessed with the use of a spectrophotometer (NanoDrop® ND-1000 UV-Vis, Wilmington, DE). For each RNA sample, measurement of the optical density at 260 and 280 nm was taken. All samples were subsequently DNase treated, in order to remove residual DNA (Ambion). 1µg RNA was converted to cDNA using a Sprint™ RT Complete Products Kit (Clontech, Mountain View, CA) and random hexamer primers.

5.2.4 Gene expression analysis

Real time qPCR was performed using a Rotor-Gene™ 6000 cycler machine. A Corbett CAS-1200 robotic bench top instrument (Corbett Life Science) was used for automated PCR setup. Each sample (15ul volume) included 5ul of diluted cDNA sample, 0.3ul primers (forward and reverse), 1.9ul of H_2O, and 7.5ul of 2X SensiMix SYBR (Quantace). All samples were tested in duplicate to eliminate pipetting errors. PCR cycling conditions and primer sequences have been described on Chapter II, section 2.8.3). Applying a normalisation strategy, when qPCR method is used, is of considerable importance, as discrepancies and false data can be generated due to sample variability (Bustin, 2005). Therefore, so-called housekeeping control genes are commonly used; the expression of these genes should be ubiquitous and similar among the experimental samples (VanGuilder, et al., 2008). Here, three commonly used housekeeping genes were used for normalisation: *Gapdh*, *Dynein*, and *β-actin* (Huggett, 2005; Isles, et al., 2004). For each sample, Ct values obtained from the three genes were averaged to provide a single normalisation value. Table 5.2.4i provides sequences for all genes. The Ct values obtained were analysed using the $2^{-\Delta\Delta CT}$ Method, as described in Chapter II, 2.8.4.2. Transformed ΔCt values, $2^{-\Delta Ct}$, were used for data analysis, as they are linear (contrary to exponential Ct values; greater $2^{-\Delta Ct}$ values denote greater gene expression).

Table 5.2.4i: Primer sequences of housekeeping and target gene used in this chapter (both Experiments 1 and 2).

Gene	Primer direction	Primer sequence
Gapdh	Forward	5’GAACATCATCCCTGCATCCA3’
	Reverse	5’CCAGTGAGCTTCCCGTTCA3’
Dynein	Forward	5’GGACATTGCTGCCTATATCAAGAAG3’
	Reverse	5’CGTGTGTGACATAGCTGCCAA3’
B-actin	Forward	5’TCTGTGTGGATTGGTGGCTCTA3’
	Reverse	5’CTGCTTGCTGATCCACATCTG3’
Sry	Forward	5’TTTCCAGGAGGCACAGAGAT3’
	Reverse	5’GCAGGCTGTAAAATGCCACT3’

5.2.5 Serum testosterone levels analysis

Upon culling, trunk blood was collected from a subset of FCG male mice (XY-*Sry*=14, XX*Sry*=7, XY=14; same mice that were used for gene expression analysis). Details on blood collection, serum storage and testosterone analysis can be found in Chapter II, section 2.6.

5.2.6 Statistical analysis

Data are presented as mean values ± standard error of the mean (SEM). When data could not be transformed, non-paramentric tests were used, as appropriate. Kruskal-Wallis was performed to test for differences in brain *Sry* expression and serum testosterone levels among the three male groups. When significant effects were obtained, subsequent pairwise Mann-Whitney tests were carried out. For the correlational analyses, Spearman's correlation coefficient was used as data deviated significantly from normality (i.e. the Kolmogorov-Smirnov test was significant); p values were adjusted using Bonferroni correction (i.e.0.05/number of tests performed) to correct for potential Type I error. P values ≤ .05 were regarded as significant.

<u>Experiment 2</u>

5.2.7 Subjects and animal husbandry

In total, an additional sixty four MF1 wildtype XY male mice were used in Experiment 2; this sample size was chosen *a priori* based upon the power to detect a two-tailed correlation of $r^2 = 0.12$, with a power >0.8, with an alpha level <0.05 (GPower, bivariate normal model). Mice were transferred from Harlan, UK, housed in cages of five, and were allowed to acclimatise at least a week prior to testing initiation (see also Chapter II, 2.1.2). Body weight was monitored regularly, especially during the water restriction schedule required to motivate performance in the maze learning task, to ensure that the mice showed no consequent ill effects.

5.2.8 Anxiety test battery

Animals were first tested on the elevated zero maze and light-dark box (three days interval between tests). Experimental conditions and measures taken for these tests were identical to those in Chapter IV (section 4.2.3).

5.2.9 Reinforcer habituation/preference test

Following the anxiety tests, animals were placed on a water restriction schedule (see Chapter II, 2.2.2) one week prior to the reinforcer habituation/preference test (see Chapter II, 2.2.3). The purpose of the former was to motivate the animals to work in the maze-based learning task. The purpose of the latter was several fold; first to habituate the animals to the reinforcer to be used in the maze task, and thereby minimise effects due to food neophobia, and second to become aware of any animals that failed to show a preference (over water) for the 10% condensed milk (Nestle, UK) reinforcer used in the maze task. The preference test occurred over 5 days and >90% of animals showed a clear preference for the reinforcer at the end of the 5 day test period. On the rare occasions, when subjects did not consume an adequate amount of the reinforcer during the 5-day testing period (i.e. >70% of total liquid consumption over the four experimental days when both liquids were available), an extra day of testing was administered.

5.2.10 Visual discrimination learning task

5.2.10i Habituation to the Y maze apparatus

Upon completion of the reinforcer habituation/preference test, subjects began training in the visual discrimination learning task using the same apparatus used previously by Lynn (2010) to show *Sry*-dependent effects on learning in the FCG model (for description of apparatus see Chapter II, 2.3.1.8 and Figure 5.2.10a below). As noted previously, during the testing period animals were maintained on a water restriction schedule to ensure motivation in performance. Habituation to the apparatus consisted of a 10-minute session. Mice were initially placed in the transparent start arm and were free to explore the maze without reinforcement in either of the goal arms. The

aim of this initiation session was to habituate the mice to the apparatus and to observe any pre-existing underlying bias in arm preference (i.e. strong preference for choosing the black or white arm). Infra-red beam breaks were recorded, which provided information on time spent in, and entries made into, each arm.

5.2.10ii Learning - acquisition phase

During this part of the experiment, animals were given a 10-trial session daily. The subjects had to learn in which of the 'choice' arms (see Figure 5.2.10a) the reinforcer was found. The reinforcer (30µl of 10% condensed milk) was always placed in the black arm for every mouse; this was to minimise any anxiety-related effects and to ensure that all mice were trained equivalently, necessary to achieve the power needed for the correlational analyses. In the beginning of each trial, the animal was placed in the transparent start arm, breaking the 280mm beam and opening all the guillotine doors. Once the mouse had moved along the transparent arm, into the centre of the maze, the 20mm beam was broken and the guillotine door of the start arm shut, to avoid return[8]. At this point, the mouse had to make a "choice" and enter one of the two goal arms; no intervention was allowed and no time limit was implemented. Upon making a "choice", the guillotine doors of the goal arms were automatically shut. The trial ended, when the mouse broke the 280mm of either goal arm and consumed the reinforcer (if there was any). It was then transferred to a holding box, where it remained during the inter-trial interval (ITI; ~65 seconds). Between trials, the maze was thoroughly cleaned with 1% acetic acid to eliminate murine odours, the reinforcer was replaced in the appropriate arm, and the location of the choice arms were pseudorandomly switched between the two possible locations of the Y configuration (Figure 5.2.10a). The acquisition phase was completed when the mouse achieved learning criterion (stable performance); this was defined as >85% correct "choices" averaged over three consecutive sessions.

[8] After a 2 minutes period, if the animal had not moved from the starting point, it was gently nudged by the experimenter into the centre and the guillotine door was manually shut.

5.2.10iii Probe session

Once criterion performance was reached, a probe session was introduced in order to test for possible odour cue usage. During this session, on trials 2,4,6,8, and 10, two previously unused black and white goal arms were used to replace those previously used. Performance on these trials would be expected to deteriorate, and even reach chance levels, if the animal was guided by odours to make a choice. After the probe session, all animals were required to re-achieve performance of >90% correct choices in a session, before completing the experiment[9].

5.2.11 Definition of behavioural measures

Infra-red placements were used in the maze to define behavioural measures (see Figure 5.2.10a for details). Each subject was considered to have made a choice when it broke the 70mm infra-red beam in one of the goal arms. Once a choice was recorded, the door of the other, non-chosen arm closed automatically. A correct choice was registered, if the animal broke the 70mm infra-red beam of the arm containing the reinforcer, and an incorrect choice (or error), if the animal broke the beam of the empty arm. Collect response latency was defined as time elapsed between breaking the 70mm infra-red beam of the chosen arm and the 280mm beam, located at the end of the correctly chosen arm.

[9] An even more stringent criterion was applied (instead of the previously used 85%) in order to make sure that mice achieved at least their pre-probe session performance levels and that potential disruption due to probe session was eliminated.

Figure 5.2.10a

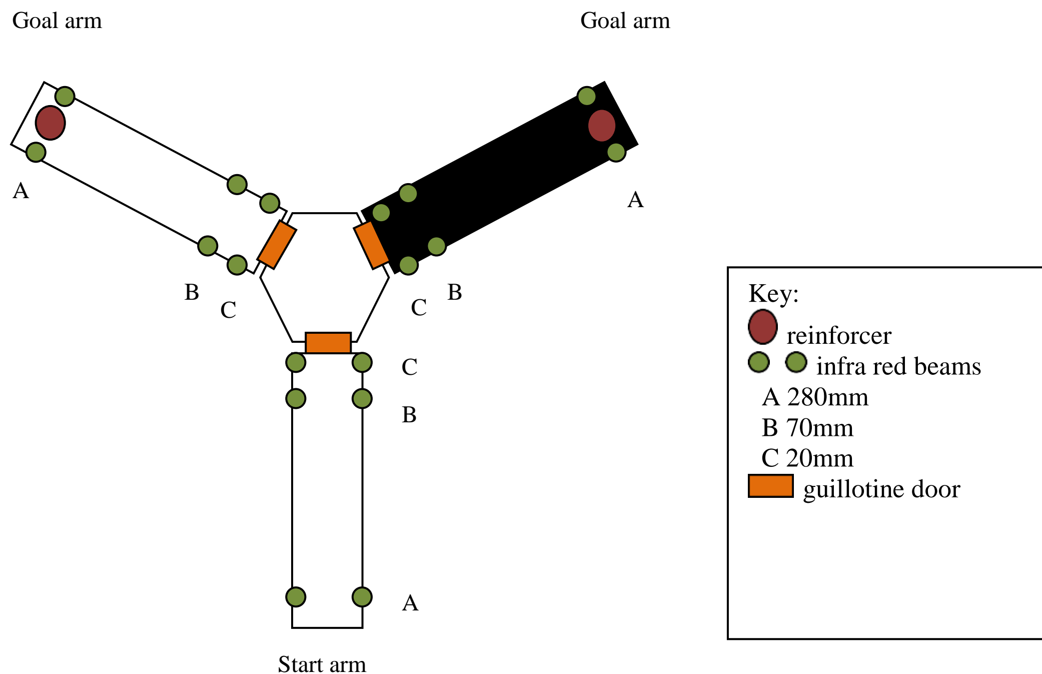


Figure 5.2.10a: Schematic diagram of Y maze apparatus. One goal arm was white and one black. The black arm was always the correct, reinforced arm. The reinforcer well could not been seen from the choice area (hexagonal space in the diagram).

5.2.12 Brain Sry expression; RNA extraction, cDNA synthesis and gene expression analysis

Upon completion of behavioural testing, sixty four XY male mice were culled by cervical dislocation and hemi-brains dissected on ice (Chapter II, section 2.7). RNA was extracted, quantified, tested for purity and converted to cDNA as in Experiment 1 (section 5.2.3). The expression of housekeeping (*Gapdh*, *B-actin* and *dynein*) and *Sry* genes was performed as described for Experiment 1 (section 5.2.4).

5.2.13 Testosterone serum level analysis

Upon culling, trunk blood was collected from 64 male mice (same mice that were used for brain tissue collection). Details on blood collection, serum storage and testosterone analysis can be found on Chapter II, section 2.6.

5.2.14 Statistical analysis and exclusion criteria

Correlational analysis was performed between *Sry* expression levels and the main putative *Sry*-dependent behavioural indices from the anxiety battery and visual discrimination learning task. These same behavioural measures were correlated with serum testosterone levels. Spearman's correlation coefficient was applied as the data deviated from normality (i.e. the Kolmogorov-Smirnov test was significant). Bonferroni correction was used to correct for multiple testing using the following formula: p value < 0.05/ total number of correlations. Regarding analysis of the two-way discrimination learning test, one animal was excluded as it was particularly anxious during testing (N=63). Learning was indexed by the total number of errors (incorrect choices) committed up to reaching performance criterion. For each experimental subject, latencies were averaged over 10 trials per session; latencies at performance criterion were given by latencies averaged over the three criterion sessions. P values ≤ .05 were regarded as significant.

5.3 Results

Experiment 1 (gonadally male mice from FCG cross)

5.3.1 Sry expression in brain

Kruskal-Wallis analysis revealed a significant difference among the three genotypes on *Sry* expression levels in hemi-brain samples (H_2 =11.347, p<.01). Post-hoc comparisons, using Mann-Whitney test, showed there was a significant difference between XY-*Sry* and XY mice (U=17, p≤.001)[10]. Specifically, male mice from the FCG cross carrying the *Sry* transgene had substantially higher (up to 30-50 fold) expression levels of the gene than wildtype XY male mice; there was no difference in *Sry* expression between the two experimental groups from the FCG cross (Figure 5.3.1a).

Figure 5.3.1a

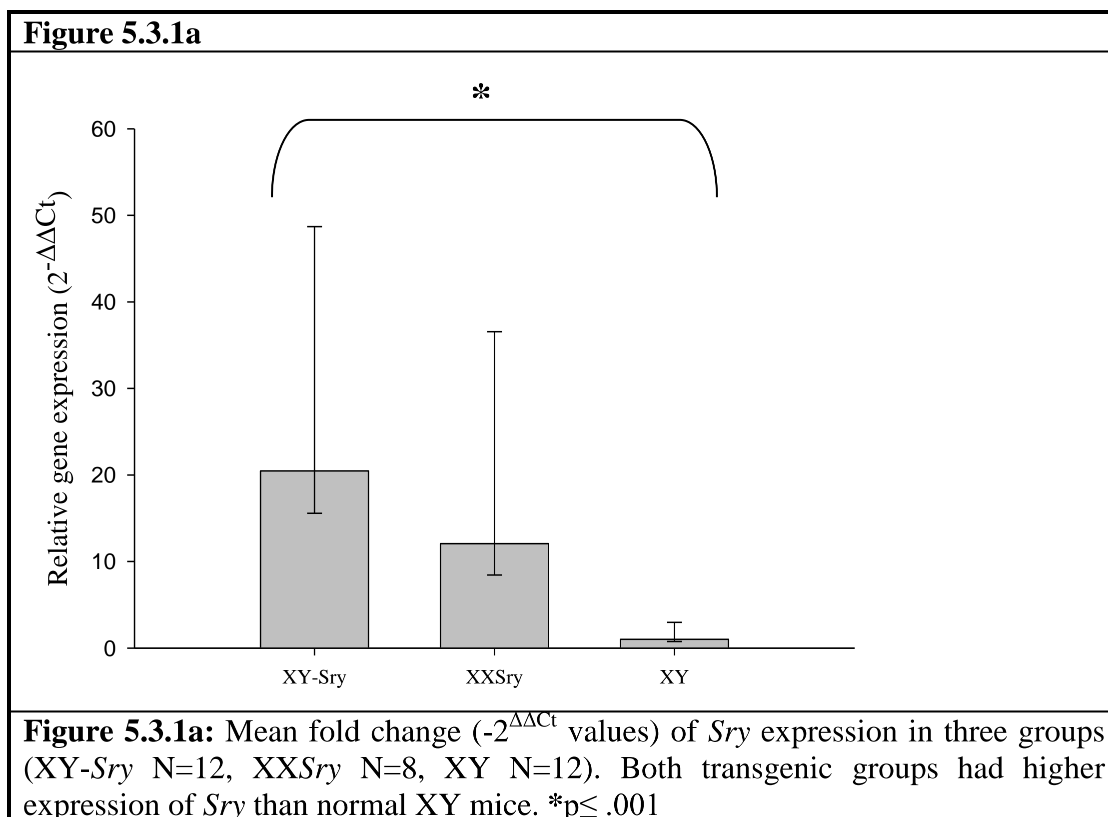


Figure 5.3.1a: Mean fold change ($-2^{\Delta\Delta Ct}$ values) of *Sry* expression in three groups (XY-*Sry* N=12, XX*Sry* N=8, XY N=12). Both transgenic groups had higher expression of *Sry* than normal XY mice. *p≤ .001

[10] Mann-Whitney analysis revealed a nominally significant difference between XX*Sry* and XY (U=11, p=0.021). However, the p value was not significant, when corrected for multiple testing (Bonferroni correction).

5.3.2 Serum testosterone analysis

Analysis of serum testosterone levels did not reveal any significant differences between the three groups (H_2=1.281, n.s.), although there was a trend for levels to be higher in XY-*Sry* mice (Figure 5.3.2a). Present testosterone levels were comparable to previous work conducted for the purpose of this thesis (see Chapter III, section 3.3.4), as well as to work conducted by others (DeVries, et al., 2002; Gatewood, et al., 2006; Samuel Barkley & Goldman, 1977; Svare, et al., 1983).

Figure 5.3.2a

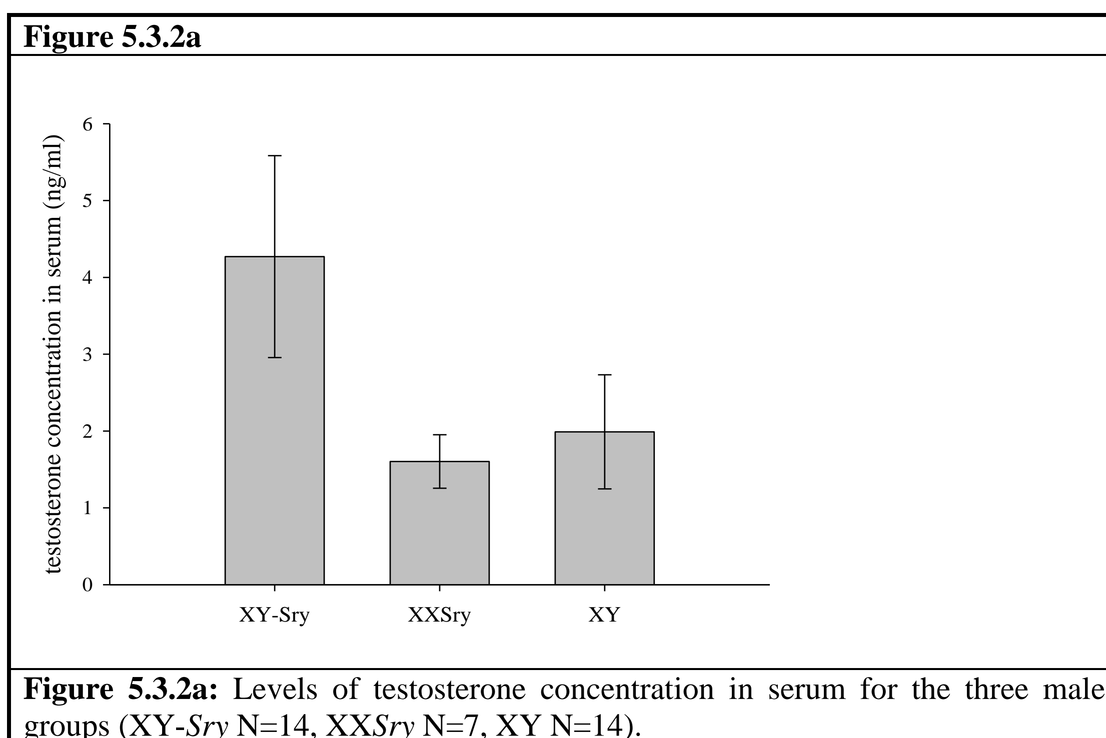


Figure 5.3.2a: Levels of testosterone concentration in serum for the three male groups (XY-*Sry* N=14, XX*Sry* N=7, XY N=14).

5.3.3 Correlational analysis with anxiety-related measures in gonadally male mice from FCG model

Brain *Sry* expression levels and serum testosterone levels were correlated with the behavioural indices shown to be affected by SRY DEPENDENCE in the FCG gonadally male mice i.e. duration of time spent in the closed arms of the EPM, total rearing in the EPM, frequency of entries and duration of time spent in the open quadrants of the zero maze, total rearing, and stretch attends in the zero maze, and latency to enter the bright area of the light-dark box. Note that, the WT XY male

group from Chapter IV were not included in the correlations below, as Experiment 2 examined correlations with behaviour (including anxiety-related behaviours and maze-based learning) in a much larger, appropriately powered cohort of normal XY males.

5.3.3.1 Correlation between Sry brain expression and anxiety-related measures

There was a nominally significant, positive correlation between *Sry* brain expression and rearing behaviour in the elevated plus maze (r= .488, p= .040, Figure 5.3.3.1a/A.). However, this value was not significant, when multiple testing corrections were taken into account (p<.0035). *Sry* brain expression was not significantly correlated with any of the other behavioural measures listed above (see Figures 5.3.3.1a and 5.3.3.1b for graphs, and Table 5.3.3.1i for summary statistics).

Figure 5.3.3.1a

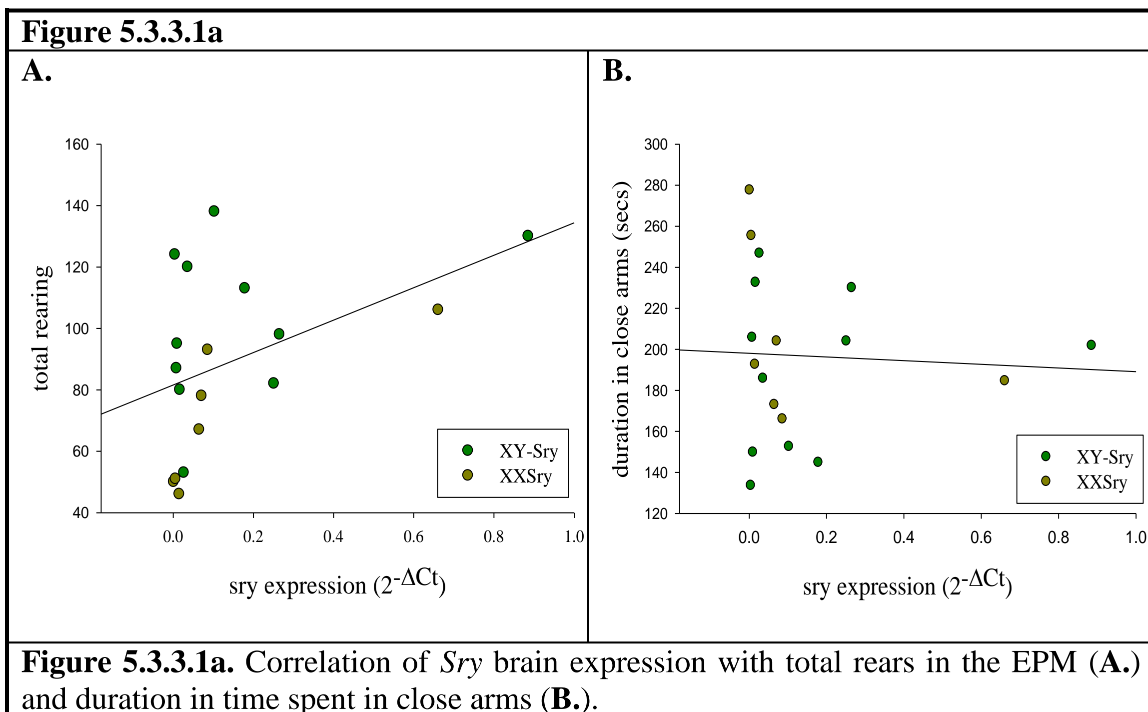
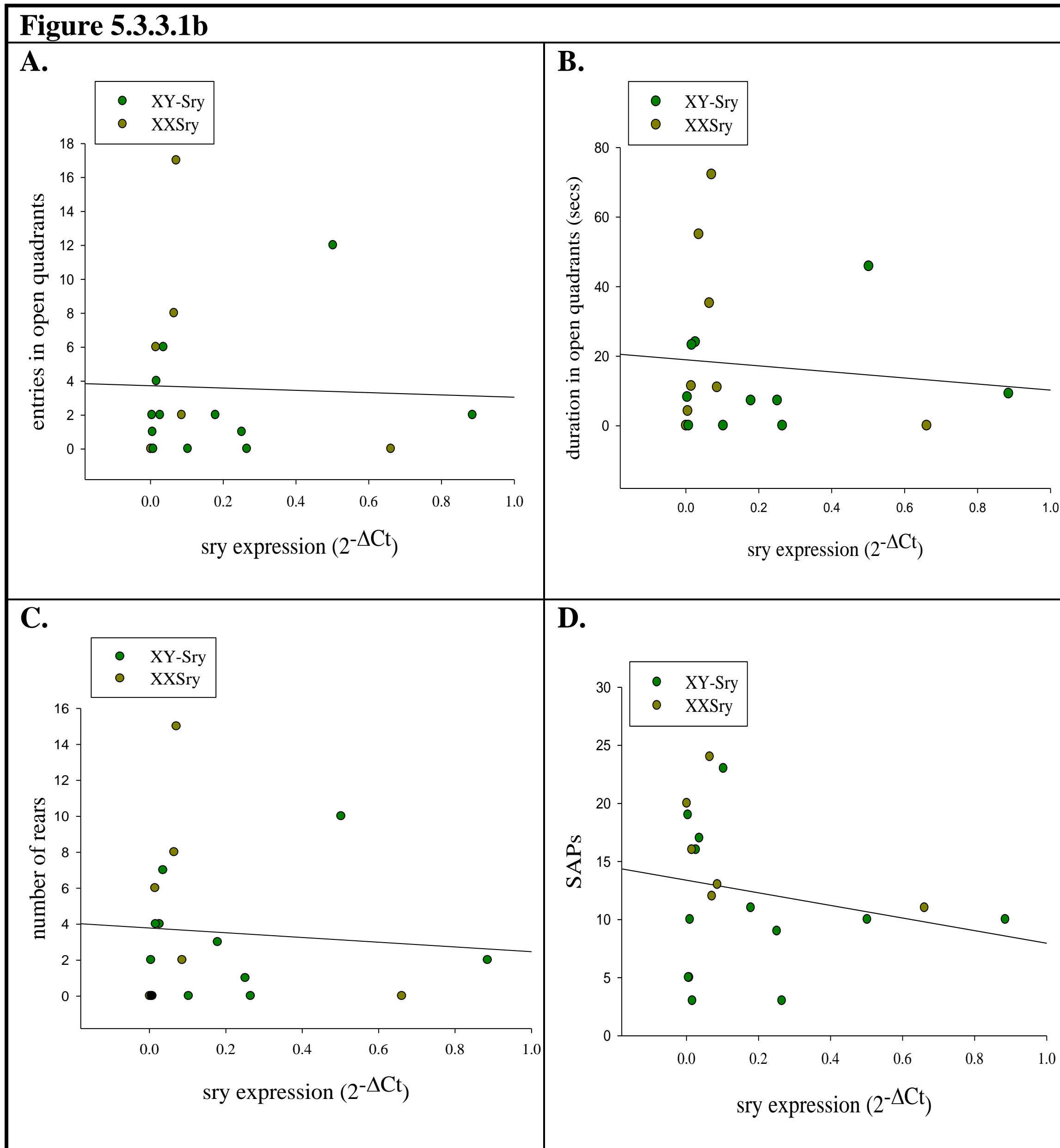


Figure 5.3.3.1a. Correlation of *Sry* brain expression with total rears in the EPM (**A.**) and duration in time spent in close arms (**B.**).

Table 5.3.3.1i: Summary of correlational analysis between *Sry* brain expression and anxiety-related behavioural measures (related to data presented in Figures 5.3.3.1a, b)

Behavioural measure	Spearman's correlation coefficient
Duration of time spent in the closed arms of the EPM	*r*= -.218, p= .385
Frequency of entries in the open quadrants of the zero maze	*r*= .012, p= .964
Duration of time spent in the open quadrants of the zero maze	*r*= .010, p= .967
Total rearing in the zero maze	*r*= .055, p= .829
Stretch attends in the zero maze	*r*= -.199, p= .414
Latency to enter bright area of light-dark box	*r*= -.181, p= .459



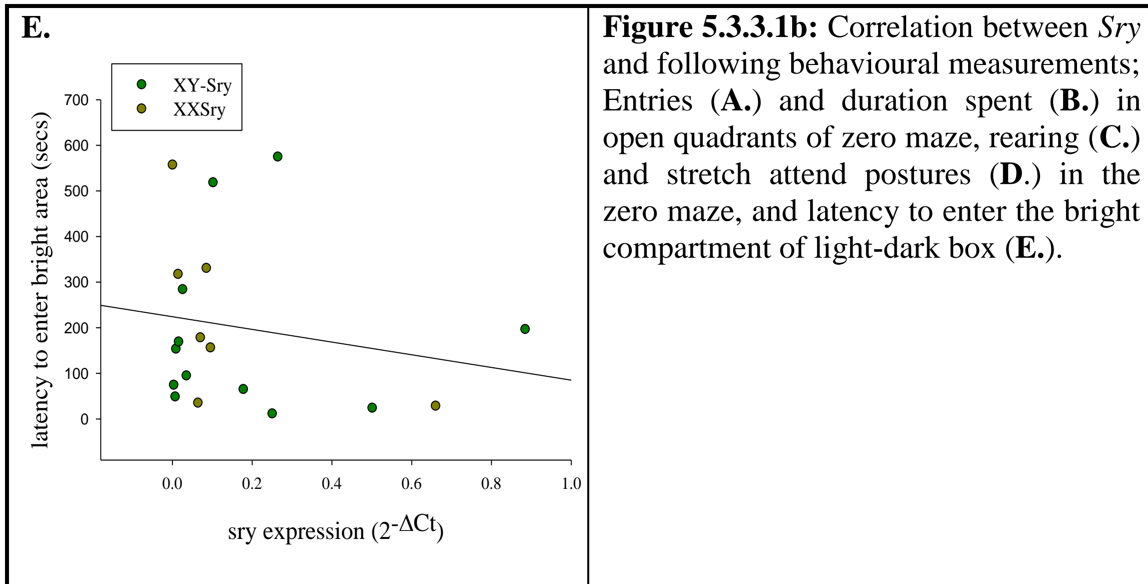


Figure 5.3.3.1b: Correlation between *Sry* and following behavioural measurements; Entries (**A.**) and duration spent (**B.**) in open quadrants of zero maze, rearing (**C.**) and stretch attend postures (**D.**) in the zero maze, and latency to enter the bright compartment of light-dark box (**E.**).

5.3.3.2 Correlation between testosterone and anxiety-related measures

No significant correlation was observed between testosterone serum levels and any of the relevant behavioural measures assayed (see Figures 5.3.3.2a and 5.3.3.2b for graphs and Table 5.3.3.2i for a summary of statistics).

Figure 5.3.3.2a

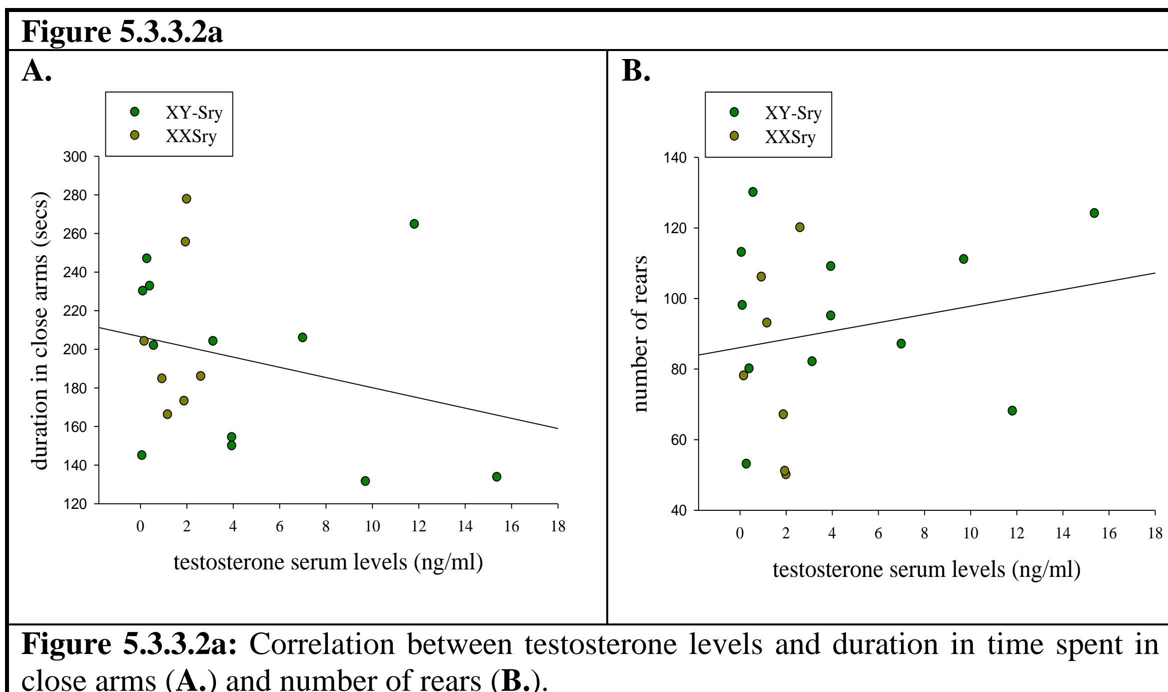
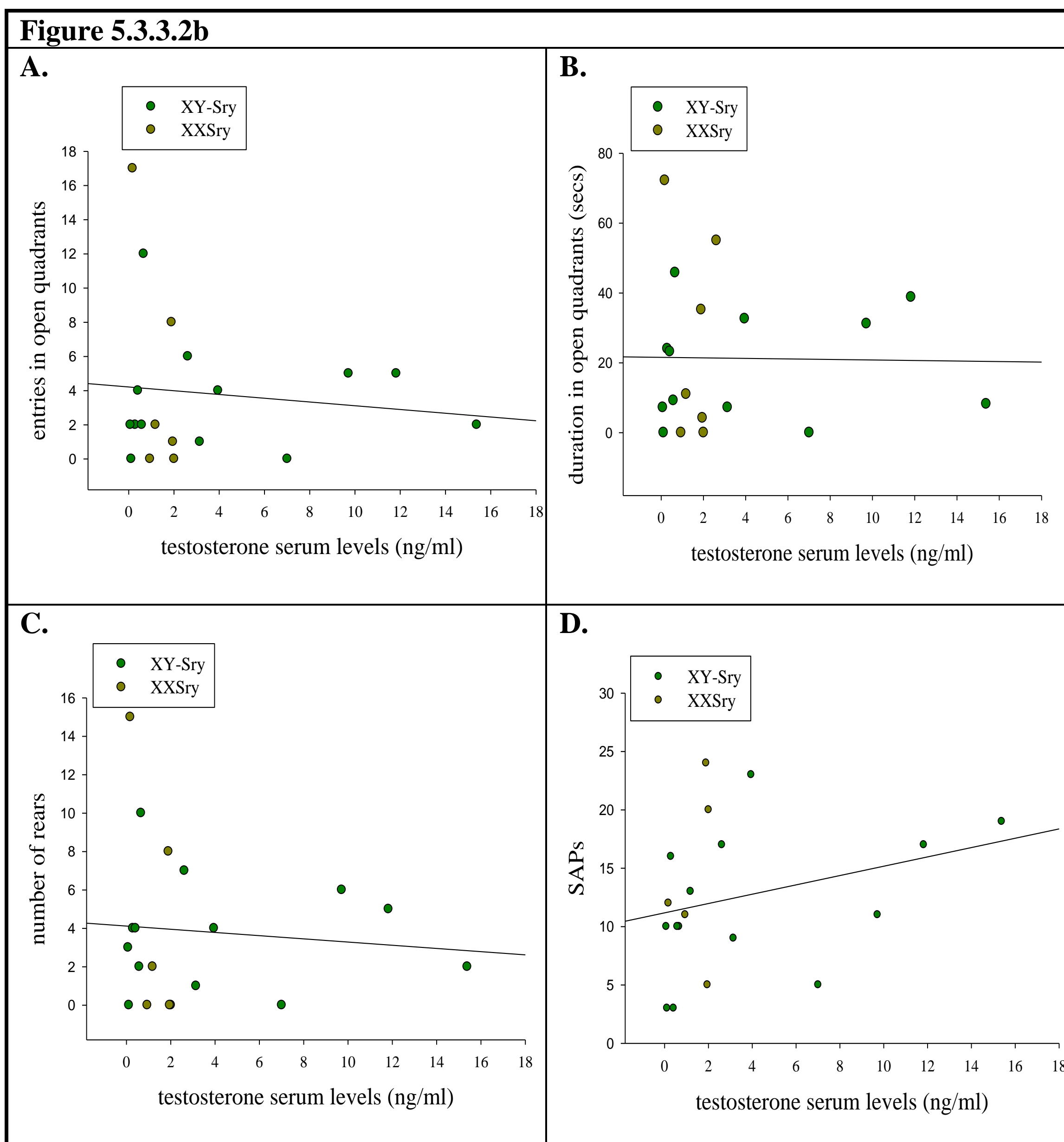


Figure 5.3.3.2a: Correlation between testosterone levels and duration in time spent in close arms (**A.**) and number of rears (**B.**).

Table 5.3.3.2i: Summary of correlational analysis of testosterone serum levels with anxiety-related behavioural parameters (related to data presented in Figure 5.3.3.2b)

Behavioural measure	Spearman's correlation coefficient
Duration of time spent in the closed arms of the EPM	*r*= -.201, p= .409
Total rearing in the EPM	*r*= .103, p= .676
Frequency of entries in the open quadrants of the zero maze	*r*= .004, p= .988
Duration of time spent in the open quadrants of the zero maze	*r*= .045, p= .855
Total rearing in the zero maze	*r*= - .055, p= .823
Stretch attends in the zero maze	*r*= .389, p= .100
Latency to enter bright area of light-dark box	*r*=- .127, p= .593

Figure 5.3.3.2b



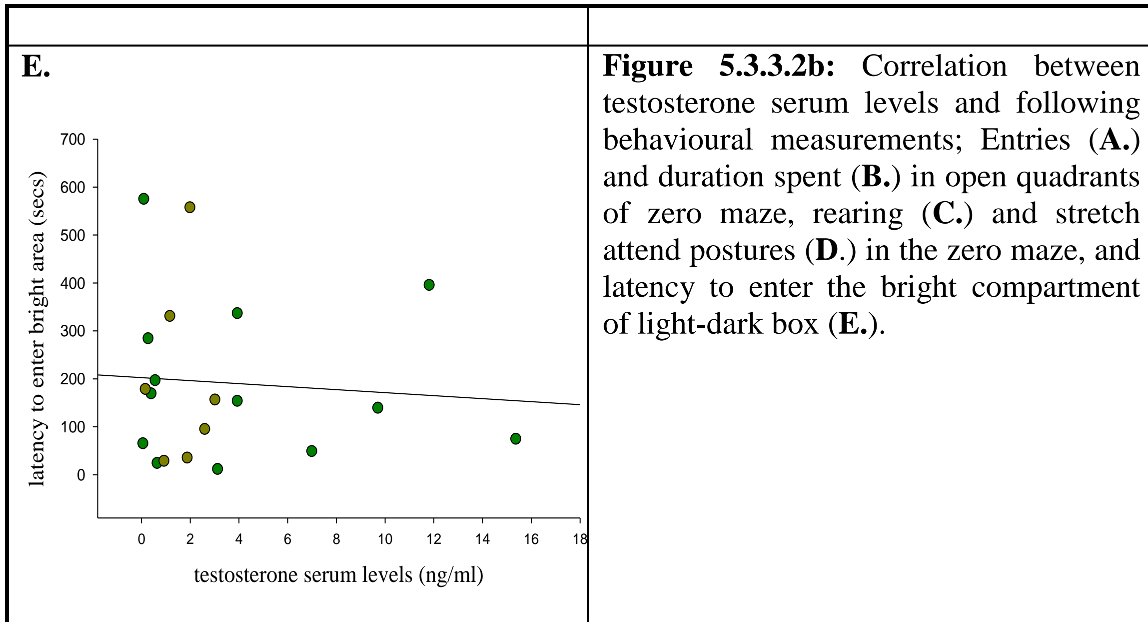


Figure 5.3.3.2b: Correlation between testosterone serum levels and following behavioural measurements; Entries (**A.**) and duration spent (**B.**) in open quadrants of zero maze, rearing (**C.**) and stretch attend postures (**D.**) in the zero maze, and latency to enter the bright compartment of light-dark box (**E.**).

Experiment 2 (additional large cohort of wildtype male mice)

5.3.1 Correlational analysis between brain Sry gene expression and behavioural measures

For correlations between hemi-brain *Sry* gene expression and behavioural measures, N = 63, as one sample failed to amplify during quantitative real-time PCR (N=62 for Y maze analysis).

5.3.1.1 Correlation of brain Sry expression with anxiety-related measures

Sry expression in hemi-brain was correlated with the following behavioural measures which had previously been suggested to be *Sry*-dependent from work on the FCG cross mice (Chapter IV): duration and time spent in the open quadrants of the elevated zero maze, number of total rears and stretch attend postures (SAPs) made in the elevated zero maze, and latency to enter the light compartment of the light-dark box. No significant correlation was reported between *Sry* expression and any relevant behavioural measure in the elevated zero maze or in the light-dark box tests (see Figure 5.3.1.1a for graphs and Table 5.3.1.1i for summary of statistics).

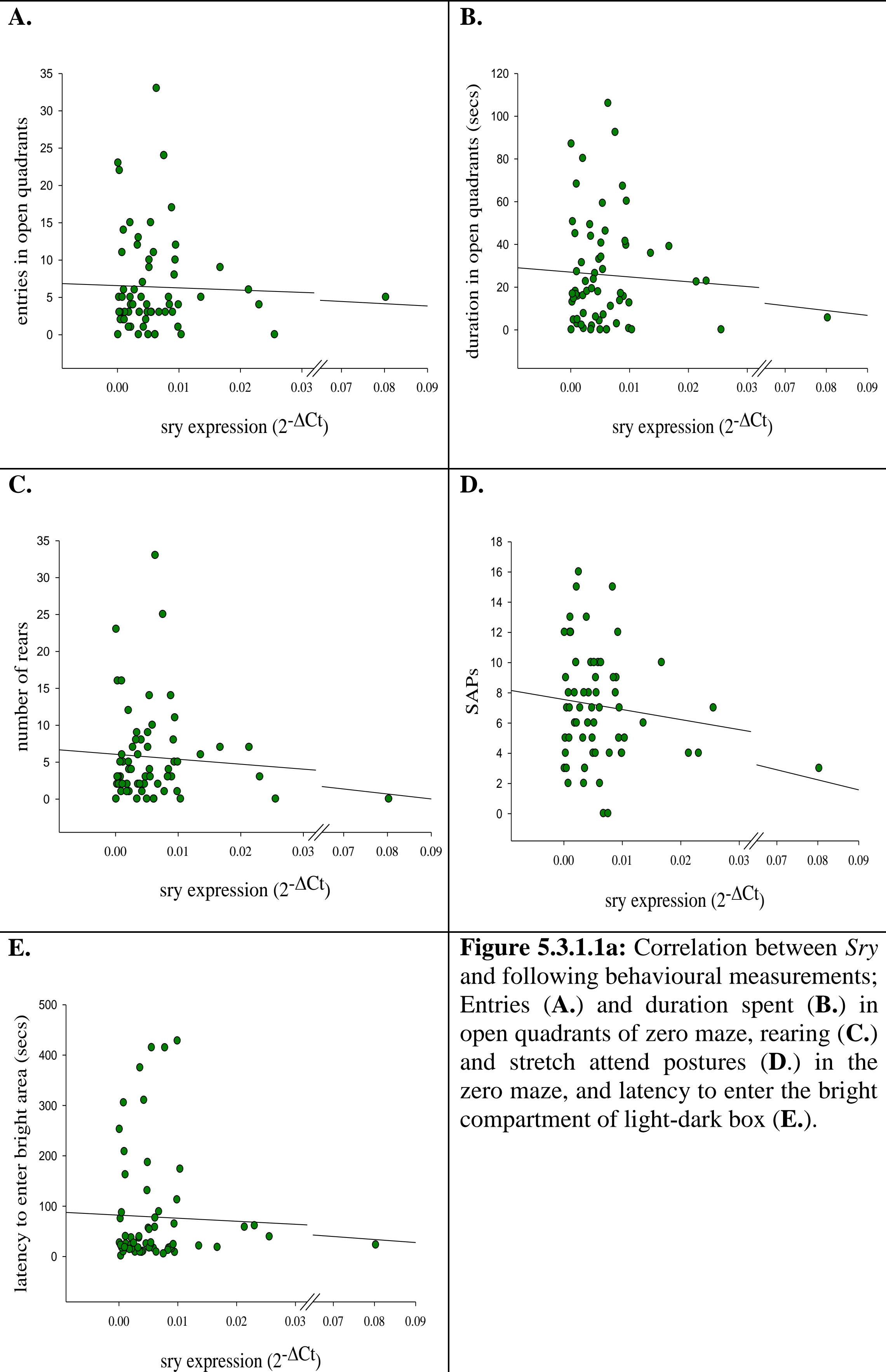


Figure 5.3.1.1a: Correlation between *Sry* and following behavioural measurements; Entries (**A.**) and duration spent (**B.**) in open quadrants of zero maze, rearing (**C.**) and stretch attend postures (**D.**) in the zero maze, and latency to enter the bright compartment of light-dark box (**E.**).

Table 5.3.1.1i: Summary of correlation analysis between *Sry* brain expression and anxiety battery measures (related to data presented in Figure 5.3.1.1a)

Behavioural measure	Spearman's correlation coefficient
Frequency of entries in the open quadrants of the zero maze	*r*= .035, p= .787
Duration of time spent in the open quadrants of the zero maze	*r*= - .010, p= .936
Total rearing in the zero maze	*r*= - .001, p= .991
Stretch attends in the zero maze	*r*= -.141, p= .271
Latency to enter bright area of light-dark box	*r*= .043, p= .739

5.3.1.2 Correlation of brain Sry expression with visual discrimination learning task measures

Measures of learning may potentially be confounded by differential reactivity to the reinforcer, or by anxiety measures (particularly given a possible mediating role for Sry in anxiety-related behaviour). Therefore, initially we tested to see whether *Sry* brain expression levels correlated with reinforcer preference or behaviour during the habituation phase of the task.

(i) Reinforcer habituation/preference test

Over the 5-day test all animals displayed an increasing preference for the 10% milk reinforcer (Figure 5.3.1.2a/A.). Correlation analysis did not reveal any significant difference between *Sry* expression levels and preference for the reinforcer (Figure 5.3.1.2a/B., *r*= .034, p= .792).

Figure 5.3.1.2a

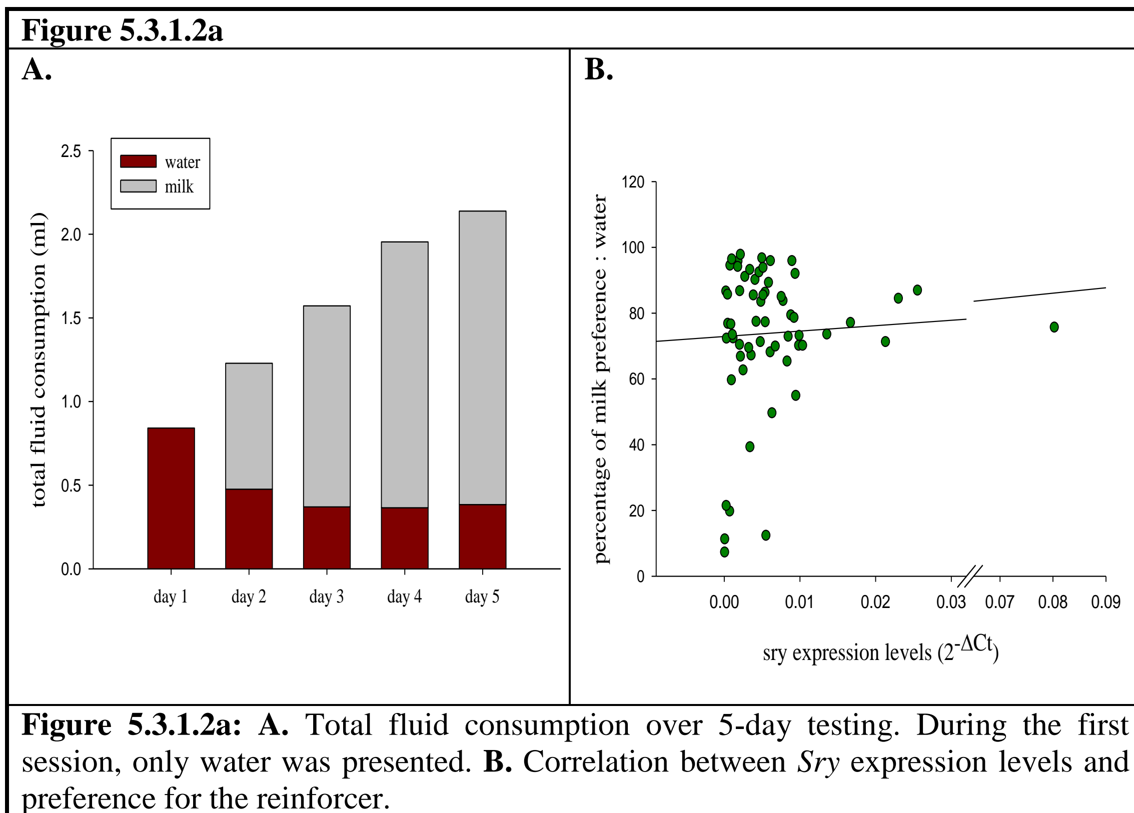


Figure 5.3.1.2a: A. Total fluid consumption over 5-day testing. During the first session, only water was presented. **B.** Correlation between *Sry* expression levels and preference for the reinforcer.

(ii) Habituation to maze apparatus

As expected, on average, animals displayed a preference towards the black arm over the white arm (mean ratio of % time in black arm: % time in white arm =1.68±.08). *Sry* expression did not correlate with this 'habituation ratio' (Figure 5.3.1.2b/A., *r*= .094,p= .472).

(iii) Acquisition of the stimulus-reinforcer contingency (errors)

Previous data from the FCG cross had indicated a *Sry*-dependent effect on learning (as indexed by total number of incorrect choices/errors) in the acquisition of a two-way visual discrimination task (Lynn, 2010). Here, we did not see a correlation between *Sry* brain expression and the same measure in normal wildtype XY male mice (*r*= -.057, p= .658, Figure 5.3.1.2b/B.).

Figure 5.3.1.2b

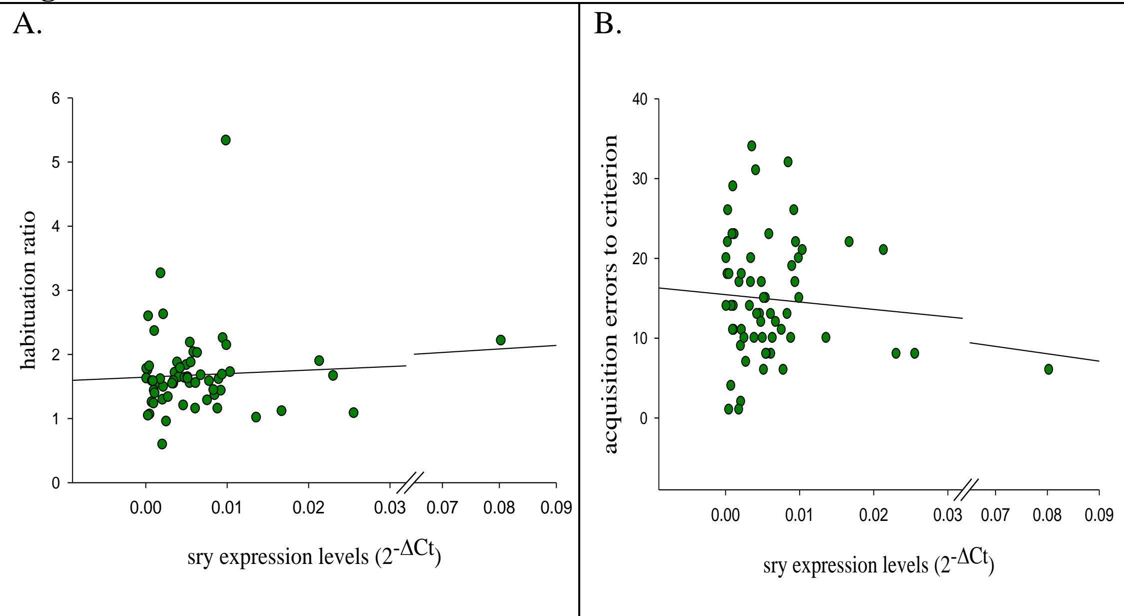


Figure 5.3.1.2b: *Sry* brain expression levels did not correlate with habituation ratio (**A.**) or total number of acquisition errors on a two-way visual discrimination task (**B.**).

(iv) Acquisition of the stimulus-reinforcer contingency (collect latency)

Sry brain expression was correlated with collect latencies over the first three sessions of acquisition and over three sessions of stable criterion performance (latencies for each mouse were averaged across ten trials per session, and then across the three consecutive sessions). No significant correlation was observed between *Sry* expression and collect latency over the first three sessions of testing (*r*= .127, p= .325), nor was there any significant correlation noted between *Sry* expression and collect latency at criterion performance (*r*= -.037, p= .777) (Figure 5.3.1.2c).

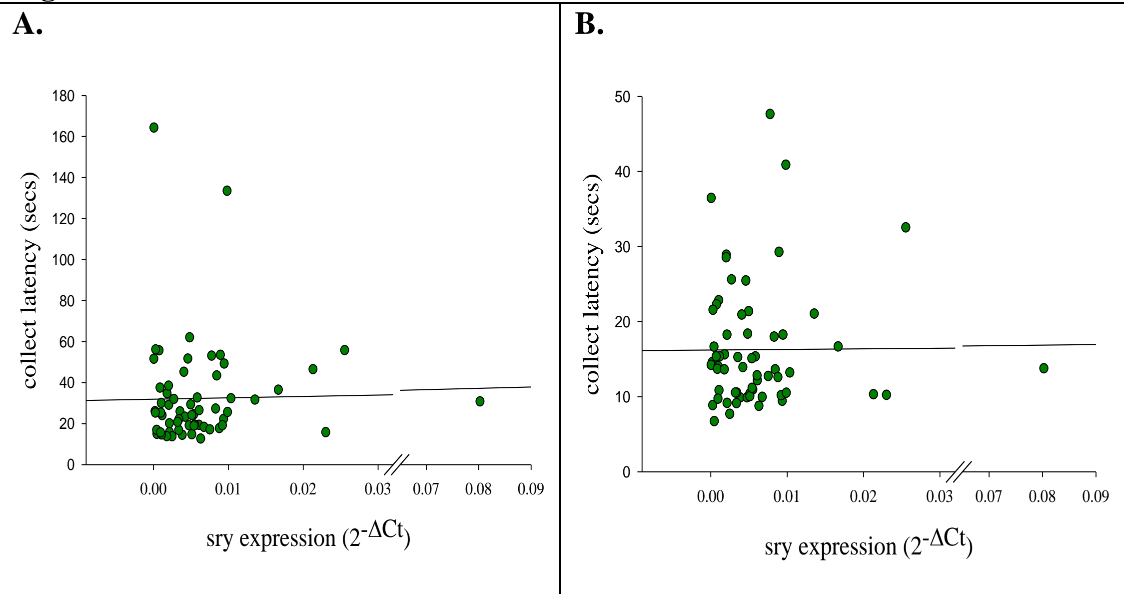


Figure 5.3.1.2c: Correlation between *Sry* and collect latencies during the first three sessions (**A.**) and at criterion performance (**B.**).

(v) Odour usage

Once subjects had reached stable performance, a probe session was introduced to assess use of olfactory cues for behaviour guiding. For each animal, the number of errors made, when the new arms were in use, was calculated (max. number equals 5). To assess whether *Sry* had any effect on odour usage, subjects were split into two groups around the median, based on their S*ry* expression levels (high/low). The average number of errors for both groups was less than one (low group: M= .48; high group: M= .65), which indicates no or little odour usage (Figure 5.3.1.2d). Pairwise Mann-Whitney test did not indicate any significant differences in errors on probe trials between the two groups (U=30.05, p= .368).

Figure 5.3.1.2d

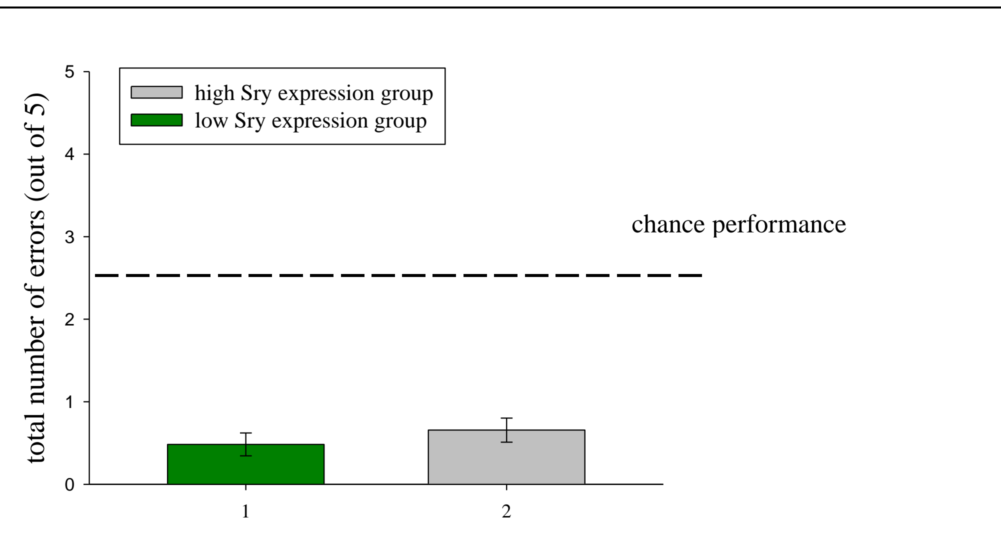


Figure 5.3.1.2d: Number of errors made by both groups on probe session. 2.5 errors would indicate that animals were performing at chance.

5.3.2 Correlational analysis between serum testosterone levels and behavioural measures

Correlational analysis was conducted between serum testosterone levels and the behavioural measures described in Section 5.3.1. Due to technical difficulties, testosterone serum levels of a subset of the experimental cohort (fifty-one mice) were used in these analyses. An *a posteriori* power calculation showed that this sample size would give a power of > 0.72 to detect $r^2 \geq 0.12$, with alpha<0.05 (GPower).

5.3.2.1 Correlation with serum testosterone levels and anxiety-related measures

No significant correlations were observed between serum testosterone levels and any of the putatively *Sry*-dependent anxiety-related behavioural measures assayed (see Figure 5.3.2.1a for graphs and Table 5.3.2.1i for summary of statistics).

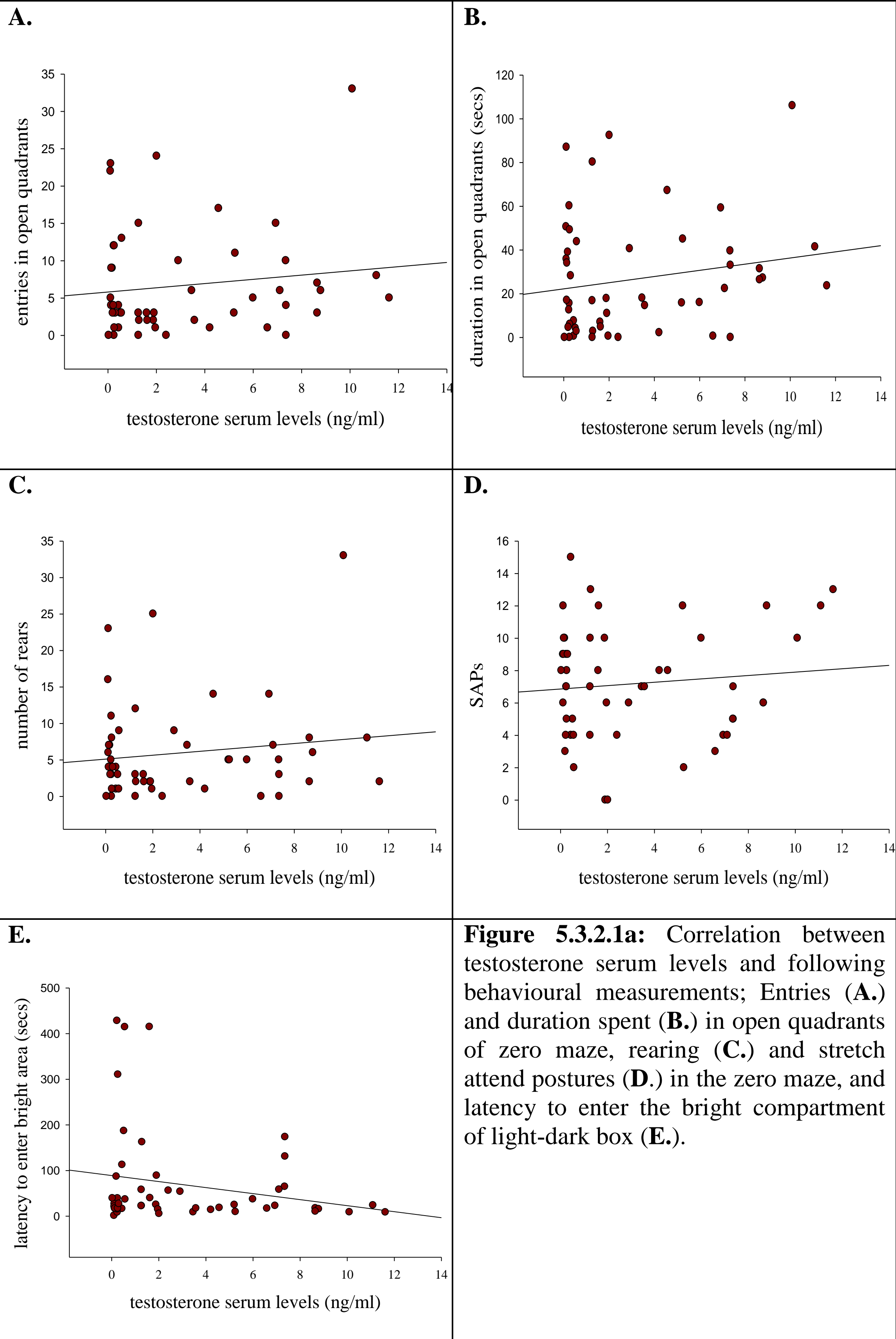


Figure 5.3.2.1a: Correlation between testosterone serum levels and following behavioural measurements; Entries (**A.**) and duration spent (**B.**) in open quadrants of zero maze, rearing (**C.**) and stretch attend postures (**D.**) in the zero maze, and latency to enter the bright compartment of light-dark box (**E.**).

Table 5.3.2.1i: Statistical summary of analysis between serum testosterone levels and anxiety-related behavioural measures (related to data presented in Figure 5.3.2.1a)

Behavioural measure	Spearman's correlation coefficient
Frequency of entries in the open quadrants of the zero maze	*r*= .073, p= .611
Duration of time spent in the open quadrants of the zero maze	*r*= .109, p= .445
Total rearing in the zero maze	*r*= .013, p= .926
Stretch attends in the zero maze	*r*= -.030, p= .835
Latency to enter bright area of light-dark box	*r*= -.120, p= .402

5.3.2.2 Correlation with serum testosterone levels and visual discrimination learning task measures

(i) Reinforcer habituation/preference test

There was no significant correlation between reinforcer preference and serum testosterone levels in the large cohort of wildtype male mice (Figure 5.3.2.2a/A., *r*= .182, p= .200).

(ii) Habituation to maze apparatus

Serum testosterone levels did not correlate significantly with ratio of % time spent in the black arm: % time in the white arm (Figure 5.3.2.2a/B., *r*= .030, p= .836).

(iii) Acquisition of the stimulus-reinforcer contingency (errors)

There was no significant correlation between serum testosterone levels and the number of errors made in reaching acquisition performance (Figure 5.3.2.2a/C., *r*= -.096, p= .504).

Figure 5.3.2.2.a

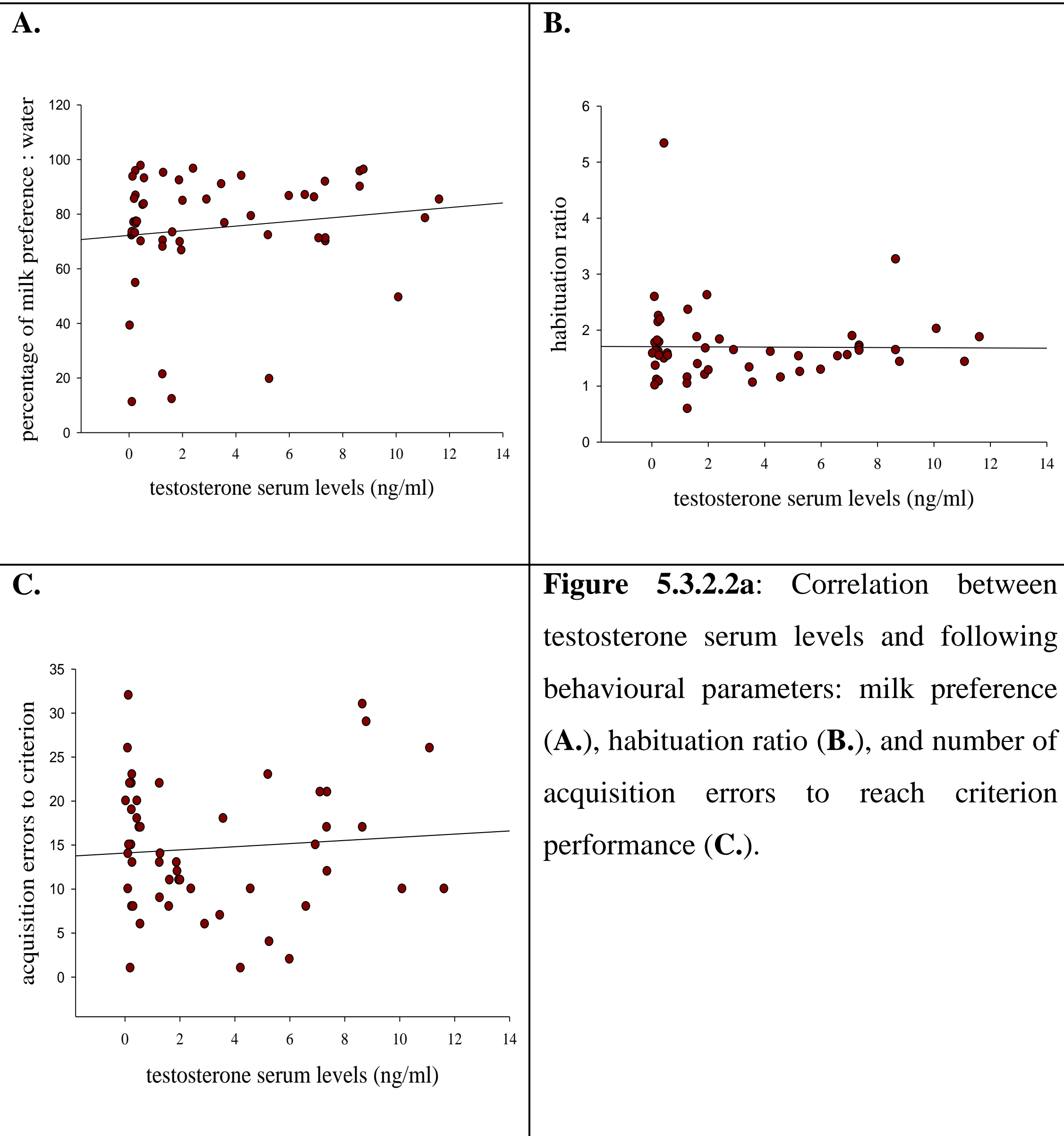


Figure 5.3.2.2a: Correlation between testosterone serum levels and following behavioural parameters: milk preference (**A.**), habituation ratio (**B.**), and number of acquisition errors to reach criterion performance (**C.**).

(iv) Acquisition of the stimulus-reinforcer contingency (collect latency)

There was a nominally significant negative correlation between collect latency over the initial three sessions of training and serum testosterone levels (r= -.361, p= .009); i.e. mice with higher levels of testosterone tended to collect the reinforcer more rapidly than those with lower levels. A similar significant negative correlation was seen across the three sessions at criterion performance (r= -.304, p= .03). Those values, though, were not quite significant, when corrected for multiple testing (p< .005) (Figure 5.3.2.2b).

Figure 5.3.2.2b

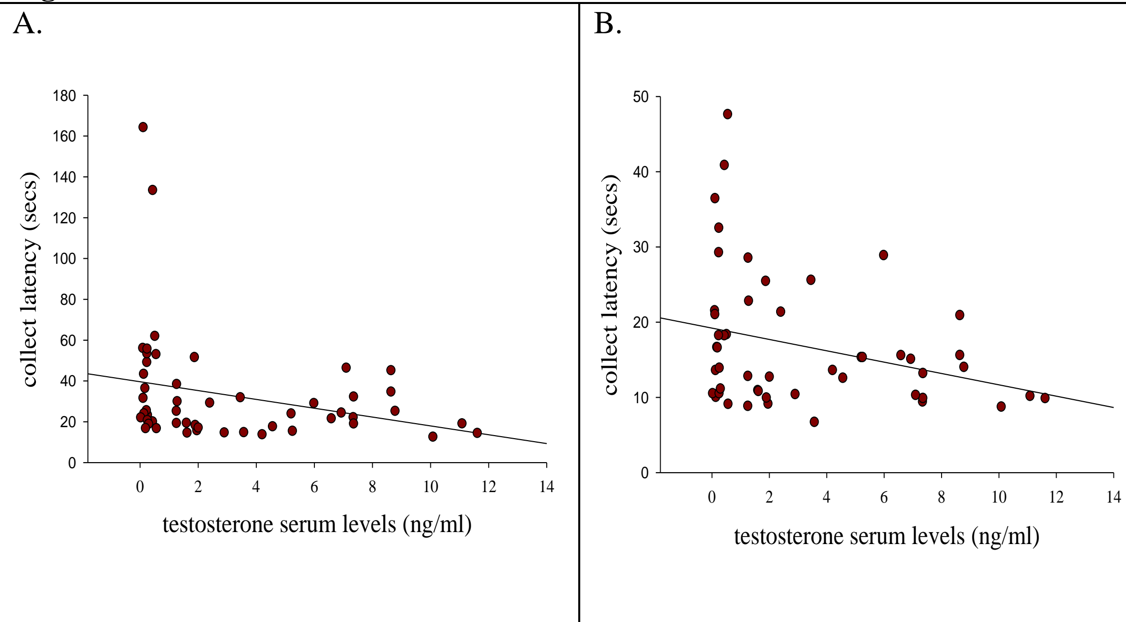


Figure 5.3.2.2b: Correlation between testosterone levels and collect latency during the first three sessions (A.) and at stable criterion performance (B.). Mice with greater levels of testosterone displayed a tendency to collect the reinforcer faster than mice with lower levels.

(v) Odour usage

Analysis of odour usage was similar to the analysis conducted for *Sry*. Subjects were split into two groups around the median, based on their testosterone serum levels (high/low). Again, the number of errors in both groups was low indicating little or no odour usage in order to make a correct choice, and pairwise Mann-Whitney test did not indicate any significant differences between the two groups (U=24.96, p= .609) (Figure 5.3.2.2c).

Figure 5.3.2.2c

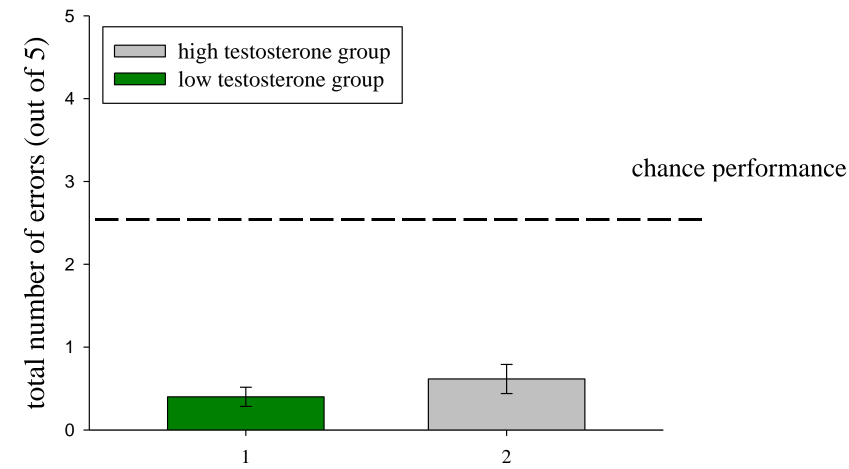


Figure 5.3.2.2c: Number of errors made by both groups on probe session. 2.5 errors would indicate that animals were performing at chance.

5.4 Discussion

Previous data in this thesis, and elsewhere (P. M. Y. Lynn, 2010), has used the FCG cross model to identify behavioural phenotypes that may be sensitive to the effects of *Sry*. Sry may potentially exert effects on behaviour through direct effects on the brain, or through indirect effects on gonadal hormone secretion. In this Chapter, we attempted to clarify the particular mechanisms through which Sry was acting to influence the individual phenotypes. To do this, we tested for correlations between *Sry* expression in the brain and putative *Sry*-dependent behavioural measures in FCG and wild type male mice; we also tested for correlations between serum testosterone levels and '*Sry*-dependent' behavioural measures in the same two groups of mice (see Table 5.4i for summary).

The majority of supposedly *Sry*-dependent behavioural measures did not correlate with *Sry* brain expression levels/testosterone levels in either FCG or wild type male mice. These negative findings could reflect several possibilities: i) an inability to detect a significant effect should there be one present, ii) some relationship between the variables that could not readily be tested using the present analyses (a 'threshold' or 'stepwise' model for example), iii) that testosterone production may be modulated by multiple factors, and therefore there is no obvious relationship between serum testosterone levels and early gonadal *Sry* expression or iv) a positive correlation between *Sry* expression in a particular brain region and the behavioural measure of interest may have been obscured by the fact that gene expression was assayed across the whole brain. We believe that the latter explanation may be the most plausible in that we used relatively large sample sizes with reasonable power to detect an effect (certainly for Experiment 2), visual inspection of the graphs revealed no obvious alternative relationship between variables and whilst there is no direct evidence that gonadal *Sry* expression may be related to testosterone levels *per se*, there is some evidence that polymorphisms within *Sry* may affect testis weight (and therefore, potentially the number of testosterone-secreting Leydig cells) (Suto, 2011). It is important to appreciate that the general strategy of correlating behavioural measures with physiological and molecular measures has been employed successfully in the past, including with regard to emotion-related behaviour (Bouayed, Rammal, Younos, & Soulimani, 2007; Laarakker, Van Lith, & Ohl, 2011).

Although the majority of findings in this Chapter were negative, we did find some evidence for a positive correlation between *Sry* brain expression and rearing on the elevated plus maze in the FCG cross male mice i.e. greater *Sry* expression was associated with a greater degree of exploratory rearing behaviour. These data suggest that the *Sry*-dependent effect on rearing, whereby gonadal males rear more frequently than gonadal females (as also described in Chapter IV) may be due to brain expression of the gene rather than downstream effects on testosterone. However, it should be noted that this correlation did not remain significant following correction for multiple testing, was somewhat disproportionately influenced by two mice, and was not recapitulated in wild type mice. Nevertheless, it might be worth attempting to replicate this finding in larger experimental groups, particularly given that rearing on the elevated plus maze might be influenced by the dopamine system, which in turn might be modulated by brain-expressed *Sry* via effects on tyrosine hydroxylase and/or monoamine oxidase A (Espejo, 1997a; Wall, et al., 2003). The lack of replication of the FCG finding in the additional large cohort of wildtype mice could feasibly be due to the larger sample size in the latter group (mitigating against a possible Type I error), or to the fact that the former group exhibit significantly higher *Sry* brain expression levels (a finding initially suggested by Lynn (2010), and replicated in this Chapter), and may therefore show a greater *Sry*-related effect.

The main positive finding from Experiment 2 was a significant negative correlation between serum testosterone level and latency to collect the reinforcer after making a choice in the visual discrimination task i.e. those mice with the highest levels of circulating testosterone were the quickest to collect the reinforcer (see Table 5.4i for summary). These data suggest that the *Sry*-dependent effect on this measure observed previously (P. M. Y. Lynn, 2010) is likely to be due to downstream effects of *Sry* on systemic gonadal hormone levels rather than to its brain expression. However, again it should be noted here that the result would not stand up to correction for multiple testing, and will require replication. Assuming the result is robust, without further specification, we can only speculate at this stage as to which precise behavioural process(es) testosterone levels may be influencing (in other words, what the ‘collect latency’ measure is indexing). Some possibilities include: reinforcer preference/motivation, anxiety, degree of odour cue usage, impulsivity, running speed or learning *per se*, although none of these unitary explanations are completely satisfactory. Arguing against an effect on reinforcer preference/motivation, we did not

see a significant correlation between testosterone levels and milk preference, nor between milk preference and collect latency (*r*= .247, p= .112); arguing against an effect on anxiety (whereby higher testosterone levels might lead to more rapid refuge-seeking in the less aversive black arm), we found no correlation between testosterone and time spent in the black vs white arm during the unreinforced habituation session, and nor did testosterone levels correlate with any anxiety-related parameters in the elevated zero maze or light-dark box. The possibility that males with higher levels of testosterone are more likely, or better able, to use odour cues to guide rapid decision-making is not supported by the fact that such males made equivalent numbers of errors to mice with lower levels of testosterone. Impulsivity is often associated with poor decision-making (Bayard, Raffard, & Gely-Nargeot, 2011; Paterson, Ricciardi, Wetzler, & Hanania, 2011). Therefore, if higher levels of testosterone were associated with higher levels of impulsivity, we might expect to see a greater number of errors in mice with high circulating levels; this was not evident here. Finally, higher testosterone levels could feasibly lead to increased motor speed; there is limited, and somewhat controversial, evidence that higher levels of testosterone influence reaction times in cognitive paradigms across a variety of species ((King, Kurdziel, Meyer, & Lacreuse, 2011; Siegel et al., 2008)**;** Muller, 1994, as cited in King, et al., 2011)). However, why this increased motor speed should only be manifest in the collection latency, and not in the start or choice latencies (P. M. Y. Lynn, 2010; data not shown) would need to be explained.

Table 5.4i: Summary of correlational analysis of Experiments 1 and 2. Note: significant results did not stand Bonferroni correction for multiple testing.

	Behavioural measure	Evidence for *Sry* direct effect	Evidence for testosterone effect
Experiment 1	Duration of time spent in the closed arms of the EPM	No	No
	Total rearing in the EPM	Yes	No
	Frequency of entries in the open quadrants of the zero maze	No	No
	Duration of time spent in the open quadrants of the zero maze	No	No
	Total rearing in the zero maze	No	No
	Stretch attends in the zero maze	No	No
	Latency to enter bright area of	No	No

	light-dark box		
Experiment 2	Frequency of entries in the open quadrants of the zero maze	No	No
	Duration of time spent in the open quadrants of the zero maze	No	No
	Total rearing in the zero maze	No	No
	Stretch attends in the zero maze	No	No
	Latency to enter bright area of light-dark box	No	No
	Preference for the reinforcer	No	No
	Habituation ratio	No	No
	Total number of acquisition errors	No	No
	Collect latency (3 first sessions)	No	Yes
	Collect latency (3 sessions of stable performance)	No	Yes

5.4.1 Summary points

- *Sry* hemi-brain expression levels are significantly elevated in transgenic male mice (XY-*Sry* and XX*Sry*) from the FCG cross relative to wildtype XY males; testosterone levels are equivalent between the three groups.
- The majority of behavioural measures previously suggested to be '*Sry*-dependent' by analyses using the FCG cross do not show an obvious relationship with *Sry* brain expression or systemic testosterone levels in FCG cross or wildtype male XY mice.
- We found some evidence for a positive correlation between *Sry* brain expression and rearing behaviour in the elevated plus maze (in FCG cross males only) and, in a large sample of wildtype XY mice, evidence for a negative correlation between systemic testosterone levels and the latency to collect the reward in the visual discrimination task. These data are consistent with the view that previously identified *Sry*-dependent effects on rearing and collect latency in the learning task may be due to brain-expressed *Sry* and downstream effects of *Sry* on gonadal hormone secretion respectively.
- Further experiments will be required in order to determine the precise nature of the behavioural processes influenced directly or indirectly by *Sry*.

Chapter VI

Developing a rodent model to test the effects of knockdown of brain expressed Sry on behaviour

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6.1 <u>Introduction</u>

In Chapter IV, work on the 'four core genotype' murine model revealed *Sry*-dependent effects on anxiety-related behaviour, as indexed by behavioural performance on the Elevated Zero Maze and Elevated Plus Maze tasks, whereby gonadally male mice (XX*Sry* and XY-*Sry*) tended to explore the more aversive areas of the apparatus more than gonadally female mice (XX and XY^{-}). This '*Sry* transgene effect' could be attributed to either: i) the expression of *Sry* acting directly in the brain, or ii) to a downstream effect of *Sry* on gonadal differentiation, hormone (testosterone) secretion and ultimately brain function. In Chapter V we attempted to use a correlational approach to begin to dissociate between these possibilities with limited success in this instance. Here, novel methods were developed in rats to address this issue more directly and determine whether reducing *Sry* expression in the male rodent brain (in the absence of any hormonal manipulations) had systematic effects on anxiety-related behaviours. If it did, this would suggest that *Sry* might be exerting its effects on anxiety behaviours through acting directly in the brain (at least in part). If not, then this might suggest that *Sry* could be exerting its behavioural effect largely via gonadal hormonal mechanisms.

We developed our rat model from one used previously. In this previous study, Dewing and colleagues (2006) used repeated (over 8 days) *Sry* antisense oligonucleotide (ODN) infusions to knock down *Sry* gene expression in the rat substantia nigra (SN). Antisense ODNs comprise 15-20 nucleotides complementary to the mRNA sequence of interest and inhibit target gene expression by halting the translation of this mRNA into protein. Ideally, the antisense ODN should be resistant to enzymatic degradation and should target specific mRNA sites (Landgraf, 1996). A common issue with the use of ODNs, especially unmodified ones, is rapid degradation by nucleases; this could constitute a problem for the successful down-regulation of the

target protein and could be avoided by various types of modification. One type of enhanced and modified ODNs is the phosphoriothioate (PS) oligonucleotides, which are used in the present chapter and are further discussed in section 6.2.3 below. Antisense experiments are usually run in parallel with control 'sense' experiments; sense ODNs have the same nucleotide sequence as the targeted mRNA and therefore should not bind to it (Kurreck, 2003). Dewing and colleagues infused unilaterally the SN with antisense ODNs and contralaterally with sense ODNs. Animals were subsequently tested in sensitive motor tasks to assess the effect of the ODN infusions on motor behaviour. Their data indicated direct *Sry*-dependent effects on molecular and behavioural measures. Downregulation of *Sry* expression levels was followed by diminished expression levels of tyrosine hydroxylase (TH), the rate limiting enzyme of dopamine biosynthesis. In terms of behaviour, animals exhibited motor deficits, resembling Parkinsonian symptoms, when using their limbs contralateral to the antisense-infused area, but not contralateral to the sense-infused hemisphere.

In our model, we decided to examine the effects of *Sry* knockdown in the ventral tegmental area (VTA) rather than the SN for the following reasons: i) VTA is, like the SN, part of the midbrain and high in *Sry* expression (Dewing, et al., 2006; Mayer, et al., 2000) ii) it is also rich in dopaminergic neurons, which, as part of the mesocorticolimbic pathway, project to many brain regions central to higher cognitive functions, including the frontal cortex, the hippocampus, and the nucleus accumbens (NAc) (Arias-Carrion, Stamatelou, Murillo-Rodriguez, Menendez-Gonzalez, & Poppel, 2010) iii) VTA is an important area for the regulation of appetitive and motivational behaviour, as well as anxiety and fear-related phenotypes (Borowski & Kokkonidis, 1996; Fields, Hjelmstad, Margolis, & Nicola, 2007; Frye & Paris, 2009), iv) lastly, research has suggested that VTA could be sexually dimorphic with regards to dopaminergic activity. Specifically, *in vitro* experiments on tissue collected from rat embryos yielded a sex difference; females had higher number of TH-ir cells in the midbrain (Beyer, Pilgrim, & Reisert, 1991) as well as higher levels of TH mRNA than males (Raab, Pilgrim, & Reisert, 1995). Furthermore, a recent *in vivo* experiment showed that the dopaminergic nuclei in the VTA of adult female rats were greater in volume compared with males (McArthur, McHale, & Gillies, 2007).We also opted to perform bilateral cannulations/infusions, mainly due to the fact that we were interested in assessing anxiety-related behaviour and there is no evidence for lateralisation of function in rodents; this meant that we required separate sense and

antisense-treated groups (i.e. within-animal comparisons as in Dewing et al (2006) were not possible). We determined the efficacy of the knockdown procedure using quantitative RT-PCR to measure *Sry* expression levels.

In developing this model, we needed to address several questions. Firstly, could the rat VTA be accurately bilaterally cannulated and infused with antisense/sense oligonucleotides in our hands, with minimal adverse non-specific effects on neuroanatomy, morbidity, health and consummatory/motoric behaviours? In order to confirm that sense infusions had no unanticipated untoward consequences on physiology and behaviour, we also examined a small group of bilaterally cannulated rats with repeated infusions of artificial cerebrospinal fluid (aCSF); data from this group are presented in Appendix C. Evaluating the motor skills of operated animals was of paramount importance, given the proximity of VTA to the SN. Intact motor skills would not only imply that the stereotaxic coordinates were correct, sparing the SN, but also that any potential anxiety-related effect was not confounded by differences in activity levels. Two motor tests were used; locomotor activity chambers and a balance beam (Brooks & Dunnett, 2009). Secondly, could *Sry* expression be significantly and stably downregulated by a repeated infusion protocol, and if so, would this also influence the expression of genes for which the Sry protein is a presumed transcriptional regulator (notably *Th*, *Maoa and Fosl1* (previously known as *Fra1*) (Cohen, Sinclair, & McGovern, 1994; Milsted, et al., 2004; Wu, et al., 2009))? Furthermore, would this impact upon the function of the dopaminergic system? Thirdly, if *Sry* expression was successfully downregulated in the VTA, would this have any impact on anxiety-related behaviours? Animals were tested on a rat version of the Elevated Plus Maze (EPM), a well validated and widely used paradigm (Pellow, Chopin, File, & Briley, 1985; Rodgers & Dalvi, 1997) which we had shown was sensitive to *Sry*-dependent effects in rodents.

6.2 Materials and Methods

6.2.1 Subjects and animal husbandry

Thirty one adult male Lister-hooded rats (Harlan, OLAC,Bicester, UK) were used in this experiment. Prior to the surgery, rats were housed two per cage. After cannulae implantation rats were housed individually until the completion of the experiment. The vivarium was maintained at 21°C with humidity levels at 55 ± 5%, and with 12:12 hour light-dark cycle (lights on at 8:00 h). Before the surgery, rats had *ad lib* access to food and water. Post-surgery, rats received daily 30g of chow to maintain their weight and had free access to water. Body weight and water consumption were monitored daily, beginning three days prior to the surgery. All experiments were carried out during the light phase of the cycle. Animal husbandry and experimental procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986. Antisense and sense-infused rats underwent the same experimental procedure, outlined in Figure 6.2.1a.

6.2.2 Surgical procedures

All animals received bilateral placement of guide cannulae into the VTA. Rats were anaesthetised with isoflurane, and placed in a Kopf stereotaxic frame (Kopf Instruments, Tujynga, CA, USA) in a flat skull position (nose bar at -3.3 mm). The scalp was incised and the skull exposed. A small hole was drilled for the cannulae using a high speed drill. Three extra holes were hand drilled on different bone plates to hold fixing screws. Bilateral stainless steel guide cannulae (26 gauge, length 11 mm below guide; Plastics One, Roanoke, VA, USA) were lowered into the VTA (AP: -5.3 mm; ML: ± 0.6 mm; DV: - 6.8 mm from bregma). Cannulae were held in place by dental cement and anchored to the skull with three fixing screws located on different bone plates. Removable dummy cannulae were inserted into the guide cannulae to prevent the cannulae from blocking. All rats received paracetamol in their water for 2 days following surgery to aid post-operative recovery. Health, weight and drinking behaviour of the rats were also monitored until the completion of the experiment.

Figure 6.2.1a

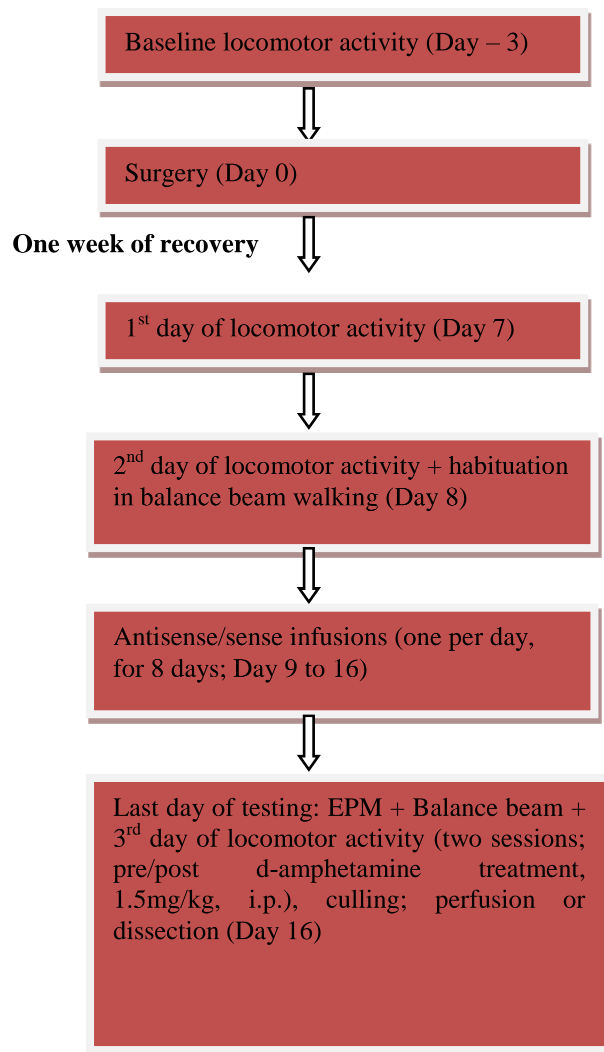


Figure 6.2.1a: Outline of the experimental design. Rats were divided into two groups; rats infused with *Sry* antisense ODNs, and rats infused with *Sry* sense ODNs. Time elapsed between surgery and behavioural testing was the same for all rats to control for possible anaesthetic effects.

6.2.3 Oligonucleotides and microinfusions

The antisense and sense oligonucleotide cocktails used for the present study were the same as those used by Dewing et al. (2006); each cocktail consisted of three ODNs corresponding to the rat *Sry* mRNA (see Table 6.2.3i). Several ODNs were phosphorothioate-endcapped; phosphorothioate (PS) ODNs are modified ODNs in which the nonbridging oxygen atom in the phosphodiester bond is replaced with sulfur. One of their advantages over unmodified ODNs is greater half-life (Kurreck, 2003). ODNs were dissolved in artificial cerebrospinal fluid (aCSF, perfusion fluid CNS, CMA Microdialysis, Sweden) to a final concentration of 1µg/µl.

Out of the twenty two operated rats (number adjusted to account for mortality rate, see section 6.3.1 below), eight rats received 8 daily infusions of *Sry* antisense ODNs, and eight received 8 daily infusions of *Sry* sense ODNs. The remaining six rats received eight infusions of aCSF (data in Appendix C). Rats were manually restrained; the dust cup and dummy cannulae were removed. Infusion cannulae (33 gauge, stainless steel, 12.5 mm in length) were implanted, extending 1.5 mm beyond the tip of the cannulae guide. The infusion cannulae were connected to two 1-µL syringes mounted on an infusion pump. A volume of 1 µL per hemisphere was infused at a rate of 1 µL/min. Following delivery the infusion cannulae remained in place for a further one minute, allowing the bolus to be absorbed. The cannulae were then removed and the dummy cannulae and dust caps replaced.

Table 6.2.3i: Base Sequences and Positions of Antisense and Sense Oligonucleotides. Phosphorothioated ODNs are in italics. Complementary triplet is underlined.

ODN	Sequence	Target region of mRNA
Antisense 1	*GCG*CTTGACATGGCCCTC*CAT*	+1 to +21
Antisense 2	*CAT*GGGGCGCTTGACATGGCCC	+5 to +27
Antisense 3	GGCCCTCCATGCTATCTAGA	-10 to +10
Sense 1	*ATG*GAGGGCCATGTCAAG*CG*C	+1 to +21
Sense 2	*AG*GGCCATGTCAAGCGCCCC*AT*	+5 to +27
Sense 3	TCTAGATAGCATGGAGGGCC	-10 to +10

6.2.4 Behavioural Methods

6.2.4.1 Locomotor Activity

Locomotor activity was assayed in locomotor activity boxes, described in Chapter II (section 2.3.2.1). Beam breaks were recorded by an Acorn 1989 computer. Each session was used as an index of subject's ability to move, reaction to novelty, and degree of habituation to novel environment. All animals were run in darkness, under the same experimental conditions, at a similar time on each day. To assess baseline motor activity, a single one hour session was performed three days prior to surgical operation. On day 7 and 8 (see Table 6.2.1i) rats were tested daily for an hour (6 bins, of 10 minutes each). On the last behavioural testing day (Day 16), rats were initially run for an hour. They were then given an i.p. injection of d-amphetamine (1.5 mg/kg), an indirect dopamine agonist (Kahlig et al., 2006; Sulzer, Sonders, Poulsen, & Galli, 2005), to ascertain possible gross effects of the experimental manipulation on the dopaminergic system, and tested in the locomotor activity boxes for an additional hour. A single, moderate, 1.5mg/kg dose was chosen, as it has been shown to sufficiently increase motor activity without leading to displays of stereotypy (Antoniou, Kafetzopoulos, Papadopoulou-Daifoti, Hyphantis, & Marselos, 1998)

6.2.4.2 Balance Beam

A narrow, wooden balance beam (1050 x 40 x 30mm, length x width x depth) with an enclosed box (300 x 210 x 160mm; length x width x height) at the opposite end was used in this experiment to assess motor function (see Chapter II, 2.3.2.2). Testing was performed in darkness, with the starting zone brightly illuminated with a 60W lamp (~300 lux) to encourage the rats to cross the beam. A line was drawn 20cm away from the start of the beam, demarcating the 'starting zone'. The beam was cleaned with 1% acetic acid between animals to minimise odours of the previous animal.

Rats were tested on two occasions; the first session on day 8 served as a habituation session. During the test, a rat was placed within the 20cm starting zone facing the box entrance at the opposite end of the beam. All trials were videotaped, and a stopwatch was used *post hoc* to measure the time taken to cross the beam. Timing commenced when an animal stepped over the starting line, and stopped once

the two front paws of the rat were placed entirely in the box. The mean of the two quickest trials was calculated. The number of footslips of each rat was also recorded.

6.2.4.3 Elevated Plus Maze (EPM)

Fear reactivity was assessed by the EPM paradigm, described in Chapter II, section 2.3.2.3. All rats were placed in the middle of the maze and were allowed to freely explore the maze for 5 minutes. Between subjects the maze was thoroughly wiped with 1% acetic acid. Behaviour was recorded and analysed *post hoc* using a computerised animal observation system (Ethovision, Noldus, NL). The maze arena was divided into five virtual zones representing the two open arms, the two closed arms, and the middle section of the maze. Data from the two open arms were combined (one cumulative zone) and data from the two closed arms were combined (a second cumulative zone).The following measures were taken for each cumulative zone: frequency of entries, time spent within the zone, distance travelled, and average velocity. Additional measures of risk assessment and exploratory behaviours including stretched attend postures (SAPs, defined as an animal keeping his hind legs in the middle or closed arm, but stretching forward the open arm), head dips (looking over the edge of an open arm) and rearing (an animal standing on its hind feet), were recorded. The principal anxiety-related measures were time spent in the open and closed arms, and entries into the open arms; entries into the closed arms and distance travelled were regarded as indices of locomotor activity (Rodgers & Dalvi, 1997).

6.2.5 Histology

At the end of the experiment, in order to assess cannulae placements in the antisense and sense-infused groups, two rats (one from each group) received a lethal dose of sodium pentobarbitone and were perfused transcardially with phosphate buffered saline (PBS) followed by 10% formal saline. The brains were removed and fixed in formal saline. Before slicing, the brains were transferred to a 30% sucrose solution in which they remained for 48 hours. Slices (40μm thick) were made using a cryostat and were mounted onto gelatine-coated slides. Slides were dried, first at room temperature, and then in the oven (40°C), before being stained with cresyl violet. Probe placement was verified with a light microscope using the atlas of

Paxinos&Watson (Paxinos & Watson, 1998). It is also of importance to note that, although, ideally, all animals should have had their probe placements verified, this was not possible due to the need to extract RNA for gene expression analyses from non-fixed tissue.

6.2.6 RNA extraction and cDNA synthesis

Upon completion of behavioural testing animals were culled by exposure to a steadily rising concentration of carbon dioxide and brain tissue from the VTA was collected (Chapter II, section 2.7).RNA was extracted from homogenised tissue (at 4ºC in a FastPrep FP120 micro-homogenizer; MB Biomedicals, U.S.) in accordance with standard protocols (Chapter II, section 2.8.1). RNA quality and concentration were assessed with the use of a spectrophotometer (NanoDrop® ND-1000 UV-Vis, Wilmington, DE). For each RNA sample, measurement of the optical density at 260 and 280 nm was taken. All samples were subsequently DNase treated, in order to remove residual DNA (Ambion). 1μg RNA was converted to cDNA using a Sprint™ RT Complete Products Kit (Clontech, Mountain View, CA) and random hexamer primers.

6.2.7 Gene expression analysis

Real-time quantitative PCR (qPCR) was performed using a Rotor-Gene™6000 cycler machine. A Corbett CAS-1200 robotic bench top instrument (Corbett Life Science) was used for automated PCR setup. Each sample (15μl volume) included 5μl of diluted cDNA sample, 0.3μl 10μM primers (forward and reverse), 1.9μl of H_2O, and 7.5μl of 2X SensiMix SYBR (Quantace). All samples were tested in triplicate to eliminate pipetting errors. PCR cycling conditions and primer sequences have been described on Chapter II, section 2.8.3. Applying a normalisation strategy is of importance in qPCR analyses, as discrepancies and false data can be generated due to sample variability (Bustin, 2005). Therefore, so-called housekeeping control genes are commonly used; the expression of these genes should be ubiquitous and similar among the experimental samples (VanGuilder, et al., 2008). Here, three commonly used housekeeping genes were used for normalisation: *Hprt*, *Dynein*, and *β-actin* (Huggett, 2005; Isles, et al., 2004). For each sample, C_t values obtained from the three

genes were averaged to provide a single normalisation value. To check that antisense and sense infusions did not elicit dissociable toxic effects on neuroanatomy, we examined the expression of three genes whose expression was known to be sensitive to mechanical damage in rodent brain: *Sst* (somatostatin), *Gat1* (GABA transporter 1) and *Plp* (proteolipid protein)(Cook, Marcheselli, Alam, Deininger, & Bazan, 1998; Rao, Dhodda, Song, Bowen, & Dempsey, 2003). We then examined expression of *Sry*, as well as four genes that could potentially be regulated by it: *Th*, *Maoa*, *Fosl1* and *Tph2*. Primer sequences for all the genes used are listed in Table 6.2.7i.

Table 6.2.7i: Primer sequences of housekeeping and target genes used in this chapter.

Gene	Primer direction	Primer sequence
Hprt	Forward	5'TTGCTCGAGATGTCATGAAGGA3'
	Reverse	5'AATGTAATCCAGCAGGTCAGCAA3'
Dynein	Forward	5'GGACATTGCTGCCTATATCAAGAAG3'
	Reverse	5'CGTGTGTGACATAGCTGCCAA3'
B-actin	Forward	5'TCTGTGTGGATTGGTGGCTCTA3'
	Reverse	5'CTGCTTGCTGATCCACATCTG3'
Sry	Forward	5'CCAGTCCTCCAAGAACCAGA3'
	Reverse	5'TAGTGGAACTGGTGCTGCTG3'
Th	Forward	5'TGTGTCCGAGAGCTTCAATG3'
	Reverse	5'GGGCTGTCCAGTACGTCAAT3'
Maoa	Forward	5'ACCAATTAATTCAGCGTCTTCCA3'
	Reverse	5'ATCATGCAGCCACAATAGTCCTT3'
Tph2	Forward	5'CTGCTGTGCCAGAAGATCATCA3'
	Reverse	5'TGCTGCTCTCTGTGGTGTCG3'
Fosl1	Forward	5'AGCCCATCGAAAGAGTAGCA3'
	Reverse	5'GATGACAACGGGTAGCACCT3'
Plp	Forward	5'ACCACCTGCCAGTCTATTGC3'
	Reverse	5'GAAAAGCATTCCATGGGAGA3'
Sst	Forward	5'CCCAGACTCCGTCAGTTTCT3'
	Reverse	5'GGCATCGTTCTCTGTCTGGT3'
Gat-1	Forward	5'GCCTGGGTGCTTGTGTATTT3'
	Reverse	5'CCACGGAAGAACAGGATGAT3'

The C_t values obtained were analysed using the $2^{-\Delta\Delta CT}$ Method, as described in Chapter II, 2.8.4.2. Transformed ΔCt values, $2^{-\Delta Ct}$, were used for data analysis, as they are linear, contrary to exponential C_t values. Data were subject to one or two-tailed t-test for independent samples as appropriate.

6.2.8 Statistical analysis

The primary comparison of interest was between antisense and sense-infused groups, and data for this comparison are reported below. A secondary comparison was performed between sense and aCSF-infused rats, in order to validate the use of the former as a control group; data from this comparison are presented in Appendix C. Data are presented as mean values ± standard error of the mean (SEM). Statistical analyses were performed using either mixed ANOVA or two-tailed independent t-test. Mixed ANOVA was used for locomotor activity data (with DAY as the within-subjects factor, and GROUP as the between-subjects factor). In addition, Analysis of Covariance (ANCOVA) was performed for locomotor activity data, with BASELINE ACTIVITY variable as the covariate (baseline motor activity levels could potentially have an effect on later locomotor performance, and were thus controlled for). Two-tailed independent t-test was used for EPM data. Data that deviated from normality were transformed where appropriate. When data could not be transformed, Mann-Whitney U-test for non-parametric data was performed. Finally, for correlation analysis, Pearson's *r* correlation coefficient was used. P values ≤ .05 were regarded as significant.

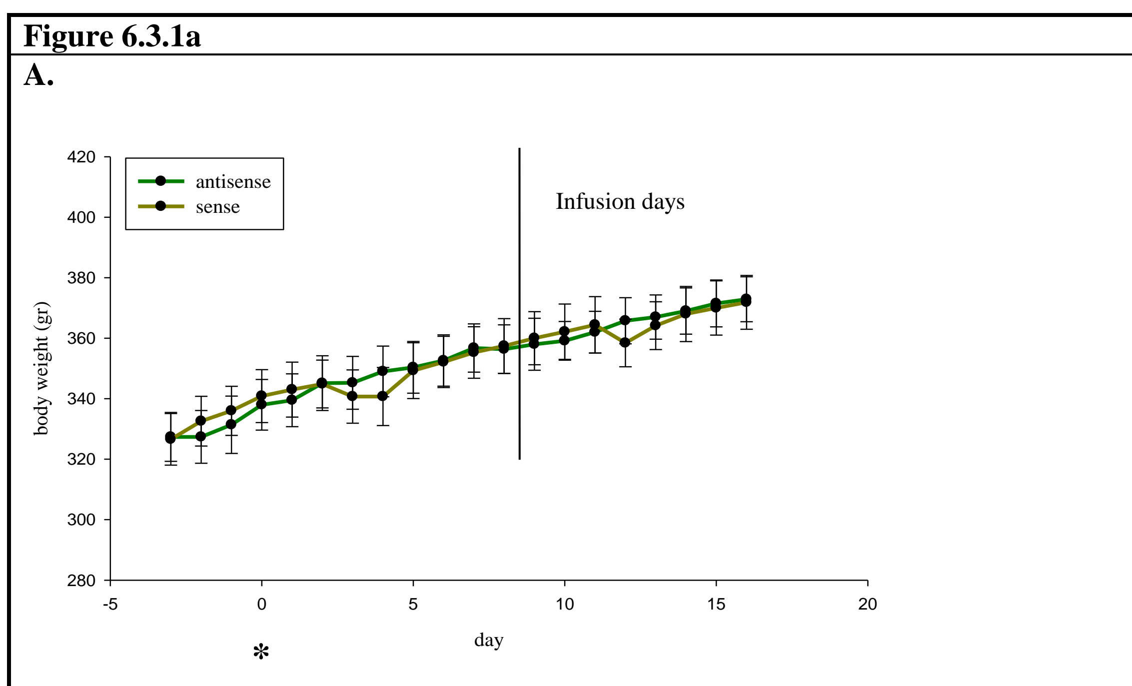
6.3 Results

6.3.1 Effects of surgery and infusions on morbidity and health

31 rats were bilaterally cannulated to the VTA in total. Of these, 6 died under anaesthesia as a result of excessive bleeding during drilling of the skull, and prior to cannulae implantation. A further two rats were culled shortly after surgery having removed their headcaps, and a third rat was culled due to illness soon after surgery (weight loss, reduced food and water intake). The remaining 22 rats were given the infusion regime described above. No obvious health issues were associated with cannulae implantation or infusions of sense or antisense ODNs. Rats in both the antisense (n = 8) and sense-infused (n = 8) groups maintained healthy body weights over the surgery and infusion days. Repeated measures ANOVA showed that the weight of all animals increased after surgery, in comparison to pre-operation weight (effect of TIME, $F_{1,14}$= 153.451, p<.01; no main effect of GROUP, $F_{1,14}$= .253, p= .623; no effect of interaction, $F_{1,14}$ = 2.338, p= .148) (Figure 6.3.1a). Furthermore, empirical observation and analysis of feeding and drinking behaviour did not indicate any significant differences between the groups (no main effect of TIME or GROUP on drinking pattern; $F_{1,14}$= 2.280, p= .153, $F_{1,14}$ = .018, p= .895 respectively; no effect of interaction,$F_{1,14}$ = 2.836, p= .114).

Figure 6.3.1a

A.



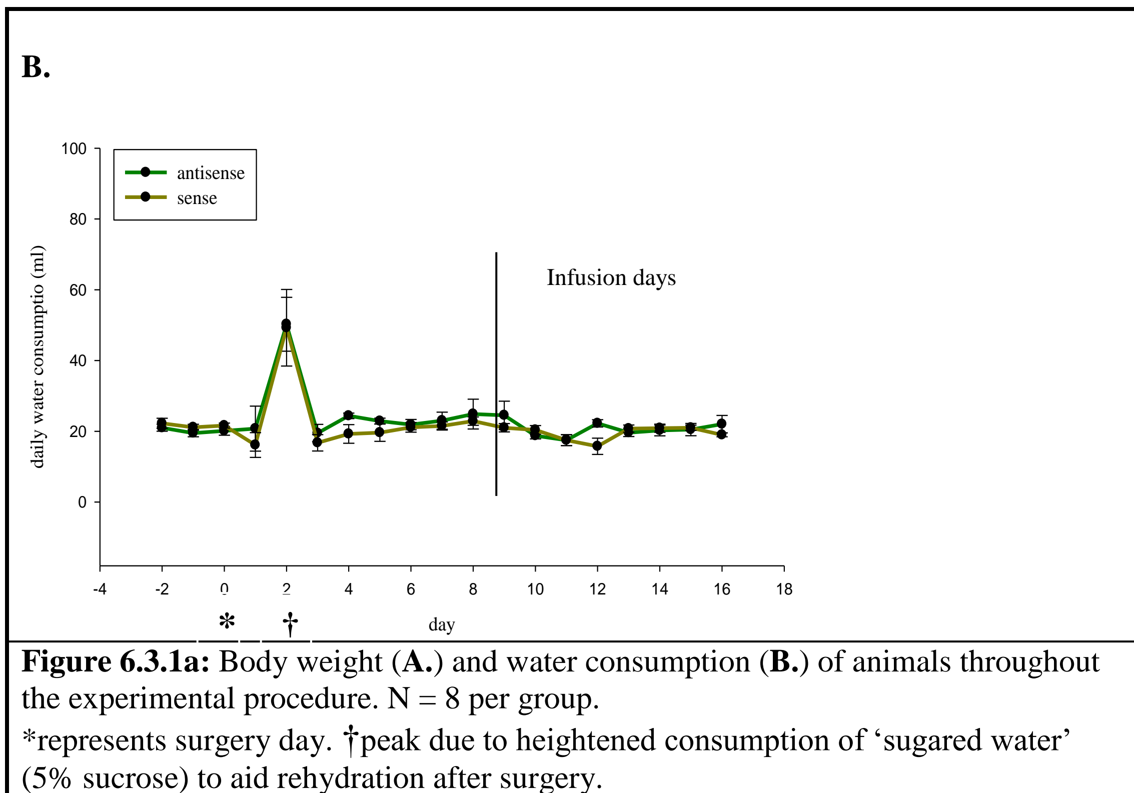


Figure 6.3.1a: Body weight (**A.**) and water consumption (**B.**) of animals throughout the experimental procedure. N = 8 per group.
*represents surgery day. †peak due to heightened consumption of 'sugared water' (5% sucrose) to aid rehydration after surgery.

6.3.2 Validation of stereotaxic coordinates

Cannulation and microinfusion sites, as anticipated, were located within the VTA, at 5.3mm from Bregma, sparing the adjacent SN; cresyl violet staining did not reveal any gross differences in brain damage in the single antisense and sense-infused rat analysed (Figure 6.3.2a).

Figure 6.3.2a

A. **B.**

C.

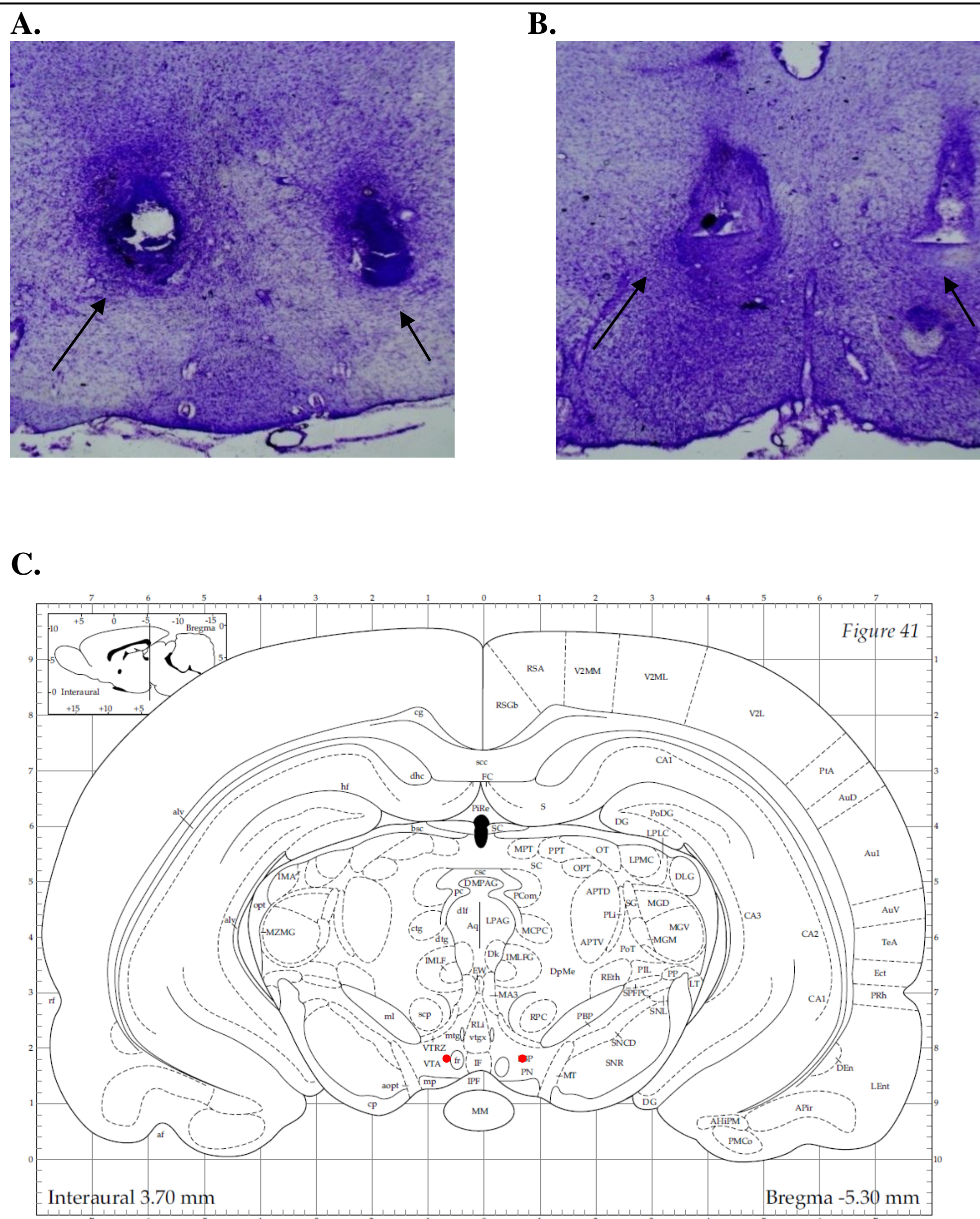


Figure 6.3.2a: Photographic image of coronal slice demonstrating the infused area of an antisense (**A.**) and a sense (**B.**) infused rat (magnification:x50). (**C.**). Representation of microinfusion sites within VTA (image reproduced from Paxinos & Watson, 1988) at -5.3mm from Bregma.

6.3.3 Gene expression analyses

6.3.3.1 Damage-related genes

The expression of *Sst*, *Gat1* and *Plp* genes was analysed in the VTA of sense (n = 7) and antisense-infused (n = 7) rats as an index of neurotoxicity. No significant between-group differences were reported with respect to *Sst* ($t_{6.974} = -1.309$, p= .232) *Gat1* ($t_{6.099}= -1.709$, p= .137) or *Plp* ($t_{12} = -.863$, p= .405) expression levels.

Figure 6.3.3.1a

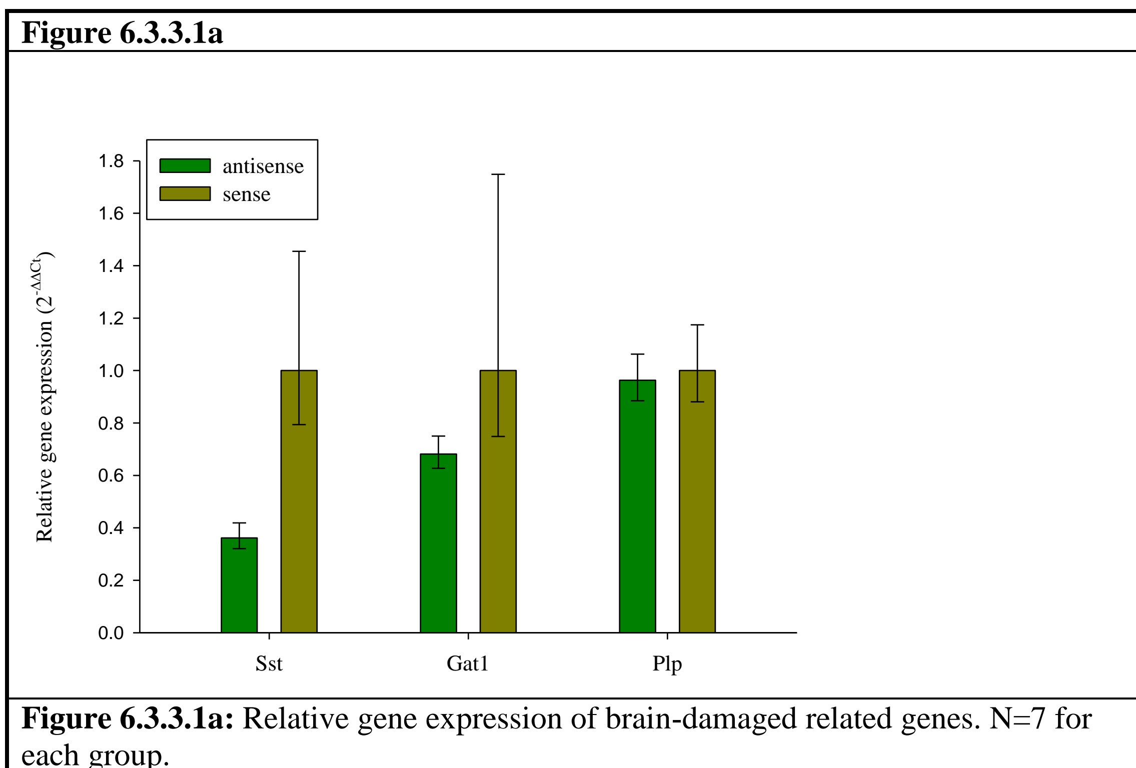


Figure 6.3.3.1a: Relative gene expression of brain-damaged related genes. N=7 for each group.

6.3.3.2 Sry and downstream genes

Sry expression levels were assessed using real-time qPCR. In addition, given the previous findings suggesting *Sry* was a transcriptional activator for *Th*, *Maoa* and *Fosl1* the expression of these genes was analysed, and their expression was correlated with that of *Sry*. The expression of a further gene involved in monoamine metabolism, *Tph2* (Walther & Bader, 2003) was also assessed and correlated with that of *Sry*. Expression of *Sry* in the antisense-infused group was lower, albeit not statistically significant, than the sense-infused group ($t_{12} = -.869$, p= .2, Figure 6.3.3.2a).

Figure 6.3.3.2a

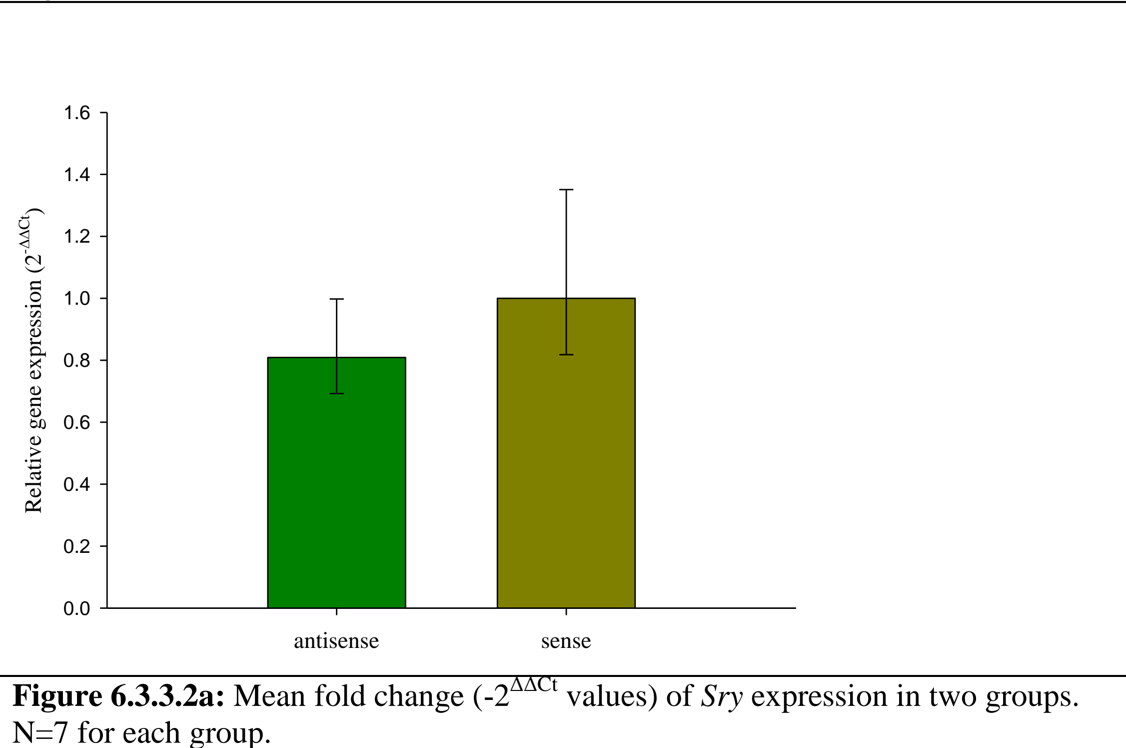


Figure 6.3.3.2a: Mean fold change ($-2^{\Delta\Delta Ct}$ values) of *Sry* expression in two groups. N=7 for each group.

No significant difference in gene expression was found between the two groups for *Th* (t_{12} = -.831, p= .422), *Maoa* ($t_{6.795}$ = -1.257, p= .25) or *Tph2* (U= 10, p= .07); however, there was a slight trend in each case for reduced expression in antisense-infused animals. Expression of *Fosl1* was significantly reduced in antisense-infused rats relative to sense-infused rats (t_{10}=-3.097, p $\leq$ .01). These data are summarised in Figure 6.3.3.2b.

Figure 6.3.3.2b

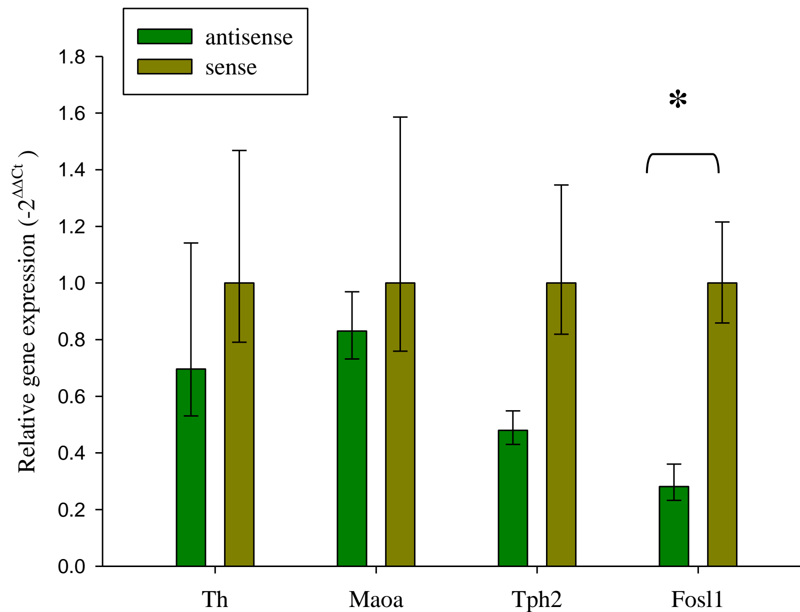


Figure 6.3.3.2b: Mean fold change ($-2^{\Delta\Delta Ct}$) of *Th*, *Maoa*, *Tph2*, and *Fosl1*. N=7 for each group (for *Fosl1*, N=6 as two samples, one in each group, failed to amplify). *p≤ 0.01.

Sry expression was significantly, and positively, correlated with *Th* and *Maoa* expression in the VTA (r = .759, p < .01; r = .782, p < .01, respectively; Figure 6.3.3.2b). In contrast, *Sry* expression did not correlate with *Fosl1* (r= .164, p= .611) nor *Tph2* expression (r_s= -.305, p= .288). These data are summarised in Figure 6.3.3.2b.

Figure 6.3.3.2c

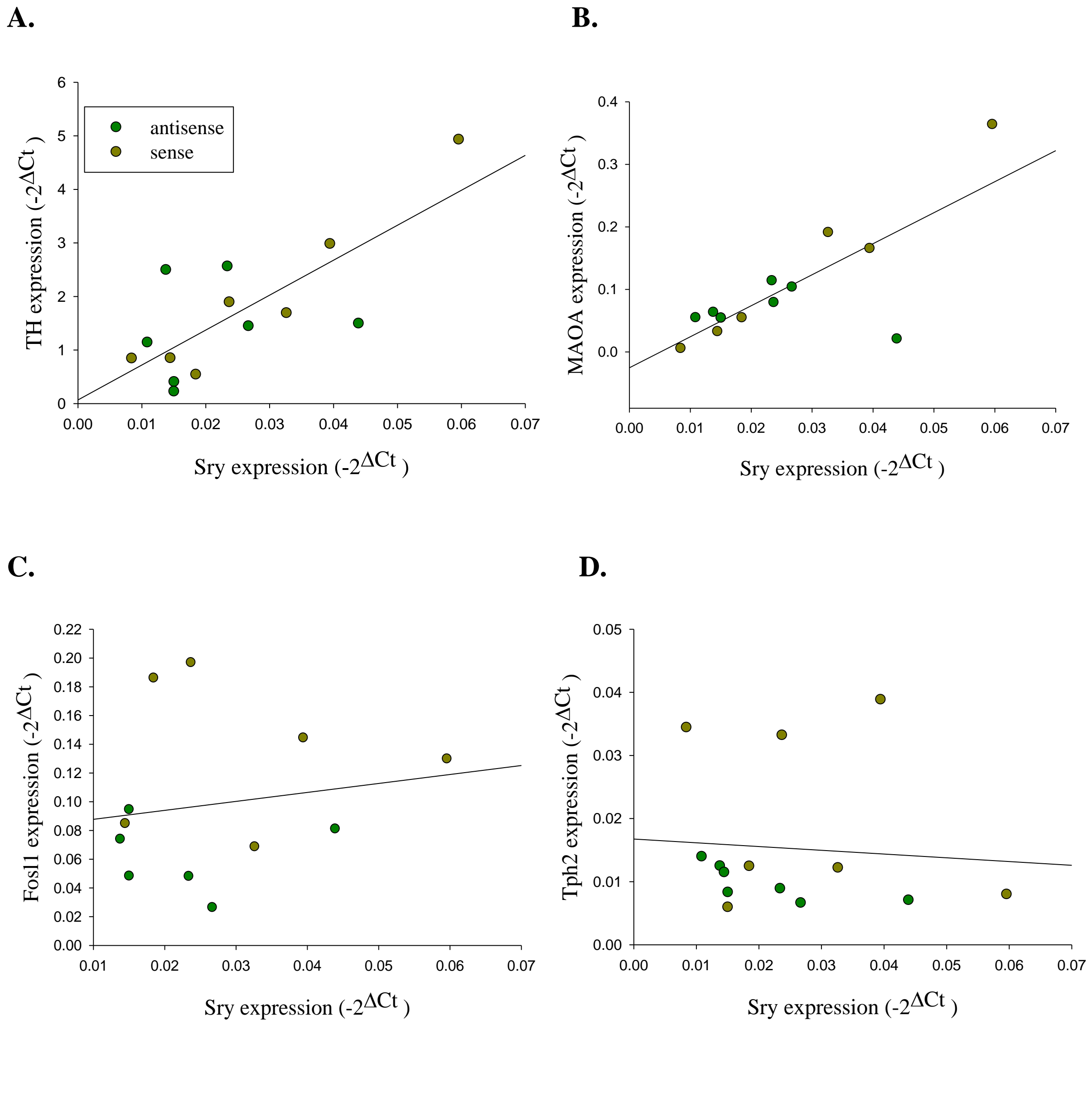


Figure 6.3.3.2c: Correlation of *Sry* expression levels with *Th* (**A.**), *Maoa* (**B.**), *Fosl1* (**C.**), and *Tph2* (**D.**).

6.3.4 Behavioural analysis

Behavioural data from rats which were perfused (but in which gene expression was not assayed) were included. Hence, behavioural data is presented for 8 rats per group.

6.3.4.1Locomotor activity analysis

There were no differences in the activity profiles of presumptive antisense and sense infused rats at baseline as indexed by beam breaks (579.1±57.6 and 629±57 respectively, t_{13} = -.612, p= .551).One animal of the antisense group was excluded from the analysis as an outlier (on day 3, the recorded beam breaks were much higher ; >2 standard deviations than the group mean, denoting either abnormal performance or failure of accurate recording). Both presumptive antisense and sense-infused groups exhibited a high degree of locomotor activity during the first testing day post-surgery; this degree of activity was slightly lower on the following day, and on the test day following the repeated infusions (although importantly rats were still moving to a significant extent) (effect of DAY, $F_{2,24}$ = 3.277, p ≤.05), consistent with both groups habituating to the novel environment. There was no overall effect of GROUP ($F_{1,12}$= .012, p= .913), nor any interaction between DAY and GROUP ($F_{2,24}$ = 2.012, p= .156). Furthermore, BASELINE ACTIVITY (covariate variable) did not interact with DAY ($F_{2,24}$ = 2.150, p= .138). Whilst d-amphetamine administration did increase locomotor activity as expected, no significant difference between antisense and sense-infused rats was observed (t_{13} = .626, p= .542). Locomotor activity data are summarised in Table 6.3.4.1i.

Table 6.3.4.1i: Performance of experimental groups in locomotor activity paradigm

Behavioural Measure	Antisense (n = 7)	Sense (n = 8)
Beam breaks, Day 0 (pre-infusions)	579.1±57.6	629±57
Beam breaks, Day 1 (pre-infusions)	539.7±52.9	618±42.3
Beam breaks, Day 2 (pre-infusions)	479.1±67.7	413.9±33.5
Beam breaks, Day 3 (post-infusions)	462±73.3	466.1±43.3
Beam breaks, D-Amphetamine(post-infusions)	524±64.0	474.5±48.5

6.3.4.2Balance Beam analysis

On the test day, both antisense and sense-infused rats crossed the balance beam rapidly (i.e. in <10s); there was no difference in performance between the groups ($t_{10.504}$ =1.006, p= .337). In general, rats did not fall or slip whilst crossing the beam. Again, there were no between-group differences on these measures (footslips: U=28, p= 1; falls: U=32, p=1).Data are summarised in Table 6.3.4.2i.

Table 6.3.4.2i: Performance of experimental groups in Balance beam walking

Behavioural measure	Antisense (n = 8)	Sense (n = 8)
Crossing time (s)	7.4±1.3	6± .7
Falls	0.1± .1	0.1± .1
Slips	0.1± .1	0

6.3.4.3 Elevated Plus Maze (EPM) analysis

The main measure of anxiety in the elevated plus maze paradigm is the time spent in the closed and open arms (and the ratio of the two).There was a significant difference between antisense and sense-infused rats on time spent in the closed compartments of the maze, with the latter group spending a greater proportion of their time in these sections (t_{14} = -2.506, p<.05). Whilst there was no significant difference between the time the two groups spent exploring the open arms (t_{14} = 1.353, p= .197) there was a trend for antisense-treated rats to spend longer exploring these compartments (p = .19). Sense-infused rats spent a significantly greater proportion of their total arm exploration time in the closed arms the antisense infused rats (44.9% vs. 19.8% respectively, $t_{10.353}$ = -2.332, p<.05). Furthermore, there were significant effects of GROUP on frequency of entries made in the closed arms, as sense infused rats made more entries ($t_{9.486}$= -2.322, p<.05). No differences were reported in the locomotor parameters of total distance travelled and average velocity (t_{14}= -.644, p= .530, t_{14}= -.218, p= .831 respectively), or number of rears (t_{14}= -.866, p= .401). Elevated plus maze data are summarised in Table 6.3.4.3i.

Table 6.3.4.3i: Performance of experimental groups on anxiety-related behavioural parameters (indexed by EPM) Significant effects of GROUP are highlighted in bold

	Behavioural Measure	Antisense (n = 8)	Sense (n = 8)	Effect of GROUP
Closed Arms	Entries	**6.5±1.0**	**12.5±2.4**	**$t_{9.486}$ = -2.322, p<.05**
	Time (s)	**43.2±11.5**	**99.6±19.3**	**t_{14} = -2.506, p < .05**
	Stretch Attends	6.2±1.5	7.2±.8	t_{14} = -.593, p= .562
Open Arms	Entries	16.9±2.4	15.1±2.8	t_{14} = 0.473, p= .643
	Time (s)	179.9±18.0	141.4±22.0	t_{14} = 1.353, p= .197
	Head Dips	18.6±2.3	15.6± 1.8	t_{14} = 1.040, p= .316

6.3.5 A comparison between aCSF and sense-infused rats

There were no significant differences between rats infused with aCSF or rats infused with sense ODN cocktail with respect to general health, consummatory behaviours, behavioural performance, or gene expression (Appendix C).

6.4 Discussion

This experimental chapter was concerned with the development and validation of a knock-down rat model in order to test for *Sry* direct effects in brain and behaviour. The strategy we used was based on one used successfully by others (albeit for a different brain region) (Dewing et al, 2006). Specific aims of the chapter were: i) to check whether the rat VTA could be successfully bilaterally cannulated and repeatedly infused with antisense/sense oligonucleotides with no major non-specific side-effects in our hands, and ii) to see whether *Sry* could be successfully knocked down with antisense ODN infusion, and, if so, to see whether this had any behavioural effects, including motor and emotional behaviours.

Using the methods described above, the rat VTA could be accurately bilaterally cannulated, as indexed by data from cresyl-violet stained sections obtained *post mortem*. However, the mortality rate associated with the surgery was relatively high (~20%), mainly due to excessive bleeding from the central sinus. In future studies, this problem may be addressed by using fibrin sponge after drilling and prior to cannulae implantation, during the surgical procedure. Repeated infusions appeared to cause minimal neurotoxicity (as indexed by damage to the VTA from brain section data) and had no gross effects on health and wellbeing (as indexed by appearance, food/water consumption and body weight). An additional important observation was that sense infusions did not have any unexpected side-effects on health, gene expression or behaviour, in that rats infused with this compound were generally equivalent to rats infused with aCSF only. Hence, the sense-infused rat represented an appropriate control for the study.

The main result from this Chapter was that, using a protocol similar to one published previously (Dewing et al, 2006), *Sry* could be downregulated by antisense oligonucleotides; however, the degree of downregulation was relatively small (~20%). Importantly, as antisense and sense-infused rats did not seem to differ in their amount of brain damage (as indicated by data from brain sections and from expression of 'brain damage-related genes') this trend towards downregulation was unlikely to be simply due to differences in cell number/function in the VTA between the two groups. Our findings contrast with those of Dewing and colleagues (2006), who reported considerable transient *Sry* knockdown using identical antisense oligonucleotides in rat brain. One potential reason for this discrepancy could be the technique applied to

measure *Sry* expression: we opted to use quantitative real time RT-PCR, a more sensitive technique than end-point RT-PCR (Schmittgen et al., 2000; VanGuilder, et al., 2008); we also examined expression in a higher number of individual animals per experimental group. Thus, we argue that our analysis method is more likely to reflect the true efficacy of antisense oligonucleotides in eliciting knockdown. A second possible reason for the discrepancy between the two studies is that the knockdown was done in different brain regions: the VTA in the present study and SN in that of Dewing et al. It is possible that the cells of the SN may be more amenable to knockdown e.g. by being more permeable to the oligonucleotides. A third reason is that the Sry knockdown might have been subject to rapid recovery, such that when the molecular measurements were made there was an underestimation of the initial knockdown (i.e. immediately following the last infusion). As the molecular determinations were done approximately 8 hours after the last infusion and given that previous data examining *Sry* knockdown in the substantia nigra (Dewing, et al., 2006) had shown a relative slow recovery of the knockdown extending over 6 days, this last possibility is unlikely. A final consideration is that our analysis of the knockdown was limited to the *Sry* transcript and a full analysis of the knockdown should include determination of protein levels.

It should be further noted that the experimental groups used here were small, and that using larger experimental cohorts may be necessary to detect subtle effects of antisense oligonucleotide administration. Alternatively, the possibility of achieving greater *Sry* reductions with the use of alternative antisense technology could be investigated. One potential way to proceed may be to use small interfering RNAs (siRNAs); although these have been shown to be able to downregulate *Sry in vitro* (Wu, et al., 2009), as well as other brain-expressed genes *in vivo* (Thakker et al., 2005; Zhang et al., 2009) they are expensive and protocols for ensuring their reliable transfection into brain cells, and their action therein remain to be optimised.

Gene expression analyses across the antisense and sense-infused groups also showed a significant, positive correlation between *Sry* expression levels and levels of *Th* and *Maoa*; functional data supporting the previously proposed notion that *Sry* acts as a transcriptional activator at the promoters of these genes (Dewing, et al., 2006; Milsted, et al., 2004; Wu, et al., 2009). Nevertheless, *Th* and *Maoa* expression did not differ significantly between the two groups, implying that diminished *Sry* expression levels were not sufficient to cause a significant alteration in these downstream genes

and therefore it was unlikely that effects on *Th* and *Maoa* were related to the behavioural changes observed in the antisense-infused animals. Tph2 expression was not significantly reduced in antisense-infused animals and there was no significant correlation between *Sry* and *Tph2* expression in VTA, implying some degree of specificity with which *Sry* regulates the expression of genes involved in monoamine function. *Fosl1* expression was significantly lower in antisense-infused rats; previous data have suggested that *Fosl1* may be transcriptionally regulated by Sry (Cohen, et al., 1994). However, there was no significant linear correlation between *Sry* and *Fosl1* expression, suggesting that *Sry* is unlikely to regulate *Fosl1* expression in a simple linear manner.

At the behavioural level, there were no significant differences between the two experimental groups in terms of motor function (locomotor activity and balance beam walking). Together with data from the brain sections, this observation indicates that the cannulation and infusion procedures did not adversely affect the function of the substantia nigra (SN), a brain region critically involved in fine motor function (Utter & Basso, 2008). In the locomotor activity cages, both antisense and sense-infused rats responded similarly to d-amphetamine treatment; d-amphetamine, a known psychostimulant, acts via dopaminergic mechanisms and leads to increased locomotor activity in animals (Antoniou, et al., 1998; Pierce & Kalivas, 1997). These data support our proposition that the dopaminergic system of the antisense-infused rats in our study remain largely intact, and imply that for *Sry* to have an effect on gross dopaminergic function, its expression levels should diminish more than 20%.

A second major finding in this Chapter was that the antisense and sense-infused groups did differ in their performance on the Elevated Plus Maze. Specifically, animals with reduced *Sry* levels spent significantly less time in the closed arms, and exhibited a trend to spend more time in the open arms, than the group with wildtype *Sry* levels. Furthermore, antisense-infused animals made fewer entries than sense-infused animals into closed arms. These behavioural differences are likely to be due to group differences in anxiety, rather than to group differences in activity for example, as there were no significant differences between the two groups in a separate activity paradigm (locomotor activity cages). Nevertheless, caution should be exercised, as closed arms entries could be regarded as variables denoting locomotor activity and not anxiety *per se* (Rodgers & Dalvi, 1997; Wall & Messier, 2001). Whilst the data from the present study suggest that *Sry* ablation in the rat VTA

might reduce anxiety (a proposal consistent with female Lister Hooded rats, which lack *Sry*, being less anxious than males on the EPM;(Johnston & File, 1991)), this interpretation should be viewed cautiously. First, as mentioned above, *Sry* levels were only marginally reduced within the antisense group. Second, the sample size in our study is small, and there is likely to be some degree of variability in probe placement, infusion efficacy and baseline anxiety levels between animals. Third, the antisense and sense-infused rats only differed on two measures (closed arm time and closed arm entries) in the EPM. Therefore, it may be worthwhile trying to replicate the current findings in the EPM, as well as investigating whether *Sry* VTA knockdown also elicits effects in other assays indexing aspects of anxiety e.g. open field and light-dark box (Prut & Belzung, 2003; Zuloaga, Jordan, & Breedlove, 2011). In terms of connecting specific *Sry*-related gene mechanisms in VTA to the changes in behaviour seen in antisense-infused animals the most likely candidate from this initial assessment would be the downregulation of *Fosl1* expression. However, there is little published data on *Fosl1* function and emotional behaviours; indeed to our knowledge *Fosl1* has previously been implicated, to some extent, only in learning and habit formation (Faure, Conde, Cheruel, & El Massioui, 2006; Gass et al., 2004).

Clearly, there are several caveats to a simple explanation of the data suggesting an influence of *Sry* on emotional behaviours including the major issue of the apparent opposite direction of effects seen in the mouse FCG model and the pattern of effects seen here in the rat model; namely an apparent anxiolytic effect of Sry from the FCG model data and an apparent anxiogenic effect of brain expressed Sry in the rat model (an issue discussed in more detail in the General Discussion that follows). Nonetheless, it may be concluded that much of the behavioural and molecular data from the present Chapter and from Chapters IV and V provide some preliminary, converging evidence for a role for Sry in mediating anxiety-related phenotypes.

6.4.1 Summary of main findings

- The rat VTA could be bilaterally cannulated with a high degree of neuroanatomical accuracy. A main practical issue related to the surgery was morbidity associated with excessive bleeding under anaesthesia.
- Repeated antisense/sense infusions caused minimal tissue damage, and were not associated with adverse effects on general indices of health and motoric

behaviours further indicating the specificity of the infusions sites to the VTA avoiding the adjoining SN.

- Sense ODN infusion had no unexpected adverse consequences (infusions elicited equivalent effects to aCSF infusions) and thus represented a valid control condition for the experiment examining antisense effects.
- Approximately 8 hours after the final infusion of antisense *Sry* was downregulated in the VTA by ~20% on average.
- In terms of molecular effects *Sry* expression levels correlated positively with *Th* and *Maoa* expression levels; however with respect to group means, the antisense infusions did not lead to any significant reductions in *Th* and *Maoa* expression levels.
- In contrast to *Th* and *Maoa*, the expression levels of *Fosl1* (another gene possibly controlled by *Sry*) were significantly reduced in the antisense-infused group.
- In terms of behaviour the approximate 20% downregulation of *Sry* expression in VTA in the antisense-infused group appeared to have discrete effects on specific components of anxiety-related behaviour as assayed in the Elevated Plus Maze.
- Overall, the molecular and behavioural findings suggest the possibility that the behavioural effects of *Sry* knockdown maybe mediated by *Sry* actions on the expression of *Fosl1*.

Chapter VII

General Discussion

7.1 Aim of the thesis

This thesis aimed to characterise the brain and behavioural effects of the Y chromosome-encoded gene *Sry* (termed *Sry*-dependent effects), as well as brain and behavioural effects underpinned by sex-linked genes other than *Sry* (termed sex chromosome complement effects). The studies presented here exploited two animal models; the murine 'Four Core Genotype (FCG) model' and a rat 'gene knockdown' model. The former model allowed for dissociation between *Sry*-dependent and sex chromosome complement dependent effects, while the latter permitted us to identify effects of *Sry* mediated solely by its brain activity. The focus of the present work was on emotional behaviours, thereby exploiting and extending work done previously in the laboratory (P. M. Y. Lynn, 2010). The data have, in particular, indicated novel relationships between anxiety and *Sry*-dependent mechanisms that may be of relevance to sex differences in brain and behaviour in rodents, and potentially also in humans.

7.2 Main findings

Chapters III to V were concerned with behaviourally defining the FCG model in more detail. We replicated *Sry*-dependent effects on anxiety-related behaviour seen previously in the laboratory (P. M. Y. Lynn, 2010), whereby gonadally male mice, possessing the *Sry* transgene (XX*Sry* and XY-*Sry*) spent more time in the more aversive regions of the elevated plus and zero mazes than gonadally female mice (XX and XY)(Chapter IV). Importantly given the well-known potential confounds in these tasks due to underlying effects on locomotor activity, this effect is unlikely to be confounded by gonadal sex differences in activity for two reasons: i) all four genotypes performed equally with regard to activity-based measures within these tasks (distance travelled and velocity), and ii) gonadally female mice displayed *higher* levels of activity than gonadal males in homecage and locomotor activity paradigms (Chapter III), consistent with previous data in rodents (Kas, et al., 2008; Koos Slob, et

al., 1986). A further finding of note from the anxiety test battery was evidence for a *Sry*-dependent effect on rearing in the elevated plus maze, and a nominally significant correlation between brain *Sry* expression and the same measure. However this finding did not survive stringent correction for multiple testing. In the behavioural screen, we also noted a sex chromosome complement effect on one measure of anxiety: specifically, XX and XY carriers differed in specific parameters of light-dark box performance (time spent in the dark and near bright area). This finding suggests that sex-linked genes other than *Sry* may be important in modulating these behavioural measures (see below).

In addition to effects on anxiety-related behaviours, *Sry*-dependent effects were noted on both bodyweight and food consumption (Chapter III), whereby gonadally male mice weighed more and consumed more food (after adjusting for body weight and metabolic scaling) than gonadally female mice. One possible mechanism for these effects is that *Sry* may modulate feeding behaviours (perhaps via effects on appetite and/or motivation). It is of note here that, *Sry* has a known controlling effect on *Th* expression and, in turn *Th* has been implicated in feeding behaviour (Sotak, Hnasko, Robinson, Kremer, & Palmiter, 2005; Zhou & Palmiter, 1995). A sex chromosome complement effect on food consumption was also identified whereby XY-*Sry* and XY- mice consumed more than XX*Sry* and XX mice. These data implicate sex chromosome linked genes other than *Sry* in feeding behaviour; there are several sex-linked genes that are known to be important in regulating appetite including *Htr2c* (encoding the serotonin 2c receptor) (Tecott, et al., 1995). As well as effects on feeding behaviour, it is important to be aware that the weight differences noted above may also have involved differences in metabolic activity, whereby the balance of calories used for metabolism and heat production may have been affected differentially in the four genotypes of the FCG model. However, to our knowledge no relevant published data using the FCG model is available.

In Chapter VI, we attempted to develop a novel rat model, by knocking down *Sry* in the VTA through the use of antisense oligonucleotides. Importantly, the VTA could be accurately cannulated and repeated infusions did not produce any serious effects on health and wellbeing of the animals. Moreover, sense-infused rats did not differ from rats infused with aCSF in any aspects of behaviour or physiology suggesting that the oligonucleotides themselves had minimal, if any, toxic effects. The

infusion of antisense oligonucleotides against *Sry* into the VTA did result in specific behavioural changes (number of entries in the close arms of the elevated plus maze) relative to sense infusions, suggesting that VTA-expressed *Sry* may be important in mediated aspects of anxiety-related behaviour. Such a proposition would be consistent with data from the FCG model. However, despite using an identical experimental knockdown protocol to one published previously (Dewing, et al., 2006), in our hands, *Sry* was downregulated by only ~20%. As discussed in detail in Chapter VI this discrepancy could be related to the different techniques applied to measure *Sry* expression (in our study, quantitative real time RT-PCR across relatively large numbers of rats; in Dewing et al, end-point RT-PCR in just one or two rats), as well as to different target areas (VTA here, substantia nigra in Dewing et al., 2006).

As noted in Chapter VI the directions of effects on anxiety were opposite when comparing Sry dependent effects revealed in the FCG mouse model to the pattern of effects seen when directly modifying *Sry* action in brain by gene knockdown in the rat model; namely, in the murine FCG model enhanced *Sry* expression seems to be associated with attenuated anxious behaviours (*Sry* transgenic mice explore the more exposed regions of the Elevated Plus/Zero Mazes to a greater extent than non-transgenic mice, and rear more in the Elevated Plus Maze), whereas in the rat knockdown model presented in Chapter VI, diminished *Sry* expression is also associated with reduced anxiety-related behaviours. This apparent paradox could be attributed to the different characteristics of the two models, including species differences. Another possibility is that the relative limited knockdown (~20%) of Sry in the rat model was not reflecting the *absence* of Sry function but was instead giving rise to some intermediate molecular (i.e. downstream gene expression effects; whereby there were no effects on *Th* and *Maoa* expression, but effects on *Fosl1* expression) and behavioural phenotypes. Additionally, the FCG model is a genetic model, where mice are born either with a *Sry* transgene or with the *Sry* gene deleted, and *Sry* acts throughout development, and throughout the brain, to influence neural function (Arnold & Chen, 2009). In contrast in the rat *Sry* knockdown model, developmentally normal adult male rats have the *Sry* gene downregulated in a specific brain region, the VTA, sparing the rest of the brain. Additionally, the *Sry* transgene could behave differently to the endogenous *Sry* gene in terms of its expression characteristics; we have shown previously that mice possessing the transgene have higher levels of *Sry* expression than mice carrying the endogenous copy of the gene

((P. M. Y. Lynn, 2010) and Chapter V data). Alternatively, the seemingly contradictory data could be explained by a 'U-shaped curve' model, whereby supra-normal (transgenic animals) or infra-normal (antisense-infused animals) *Sry* expression levels cause similar disruptions to neurobiology, and hence exert similar effects on anxiety-related behaviours.

7.3 Potential mechanisms of Sry action

Sry can exert its effects either directly or indirectly (via gonadal hormones). Where the behavioural data from the FCG model indicated a *Sry*-dependent effect on emotional functioning, an attempt was made to dissociate between these two possibilities by correlating *Sry* brain expression and serum testosterone with behavioural performance. Besides the nominally-significant correlation between rearing behaviour in the elevated plus maze and brain-expressed *Sry* referred to above, the results of these correlations were negative. In the case of the FCG data, the 'n' was, in terms of that required for a comprehensive correlational analysis relatively small. Hence, these data could have suffered from a relative lack of power. Another possibility for the lack of correlation between behaviour and serum testosterone levels in particular, was that *Sry* may have been exerting downstream effects via steroid hormones other than testosterone, e.g. via dehydroepiandrosterone/DHEA, an adrenally produced steroid, precursor of testosterone and estradiol; (Labrie, Belanger, Simard, Luu-The, & Labrie, 1995) which we did not measure.

A lack of power was less likely to have been the explanation for any general inadequacy of the correlational analysis attempted with the large group of wildtype XY males in Experiment 2 of Chapter V, made in an attempt to determine the physiological relevance of the findings made in the FCG model (see below for further discussion on implications for physiological relevance to sex differences in behaviour). However, as with the smaller 'n' from the FCG model, there were very few significant correlations between behaviour (in this case both anxiety behaviours in the EPM and learning in the appetitive motivated maze-based visual discrimination task) and either direct *Sry* effects (those related to *Sry* expression levels in brain) or indirect *Sry* effects (those related to serum testosterone levels). Given the generally negative data obtained across most of the correlational analysis it may be the case that, in this instance, the correlational analyses were not sensitive enough to capture

the complexity associated with multiple interacting gene and hormonal factors modifying behaviour. However, it is not the case that this correlation analysis strategy in general is without value, as it has proved informative in several contexts in previous studies (Bouayed, et al., 2007; Laarakker, et al., 2011) and it may prove more fruitful in our hands in future work where we to measure, for example, the levels of other gonadal hormones, or measure gene expression/testosterone levels at different developmental time-points.

In contrast to the FCG mouse model and associated correlational analyses, the data from the *Sry* knockdown model in the rat did give rise to some interesting possibilities for molecular mechanisms underlying the action of brain expressed *Sry* on behaviour. As discussed above, we found that antisense-treated rats showed patterns of behaviour consistent with reduced levels of anxiety and showed significant downregulation of *Fosl1* (a gene previously shown to be regulated by *Sry*), but no significant downregulation of key genes associated with dopaminergic function (i.e. *Th* and *Maoa*). Whilst preliminary, these data might suggest that anxiety-related behaviours resulting from the manipulation of *Sry* expression might be mediated by *Fosl1* activity in the VTA rather than by altered dopaminergic function in the same region. *Fosl1*, an immediate early gene (IEG), is part of the Fos related antigen proteins (along with *c-fos* and *Fosl2*) and a component of the transcription factor AP-1 (activator protein-1) (Foletta, 1996). AP-1 proteins regulate many cellular processes and Fosl1, in particular, has been implicated in tumorigenesis, cancer growth, and osteoclastogenesis (Bakiri et al., 2007; Young & Colburn, 2006). The exact function of *Fosl1* in the brain is not well-known, as *Fosl1* 'knockout' mice die in utero from developmental deficits. Nevertheless, *Fosl1* has been shown to successfully replace specific *c-fos* functions during development (Gass, et al., 2004).

7.4 Implications for sex differences

The main findings of the current thesis could provide insight into sexual dimorphisms in wildtype rodents, as well as humans. Work from the FCG model suggests that *Sry* could regulate anxiety-related behaviour (anxiolytic effects), activity (reduction), and food consumption (increase). In terms of activity and food consumption, previous literature on wildtype animals has suggested that i) females are indeed more active than males in some behavioural paradigms (Blizard, et al., 1975; Koos Slob, et al.,

1986; Tarantino, et al., 2000) and ii) males consume more food and weigh more than females (Bell & Zucker, 1971b; Koos Slob & Van Der Werff Ten Bosch, 1975b). In contrast, research on anxiety-related behaviour is more complicated. Behavioural assays on anxiety might not tap on the same parameters and/or underlie same neurobiological mechanisms (Belzung & Le Pape, 1994; Griebel, et al., 2000). This notion was highlighted in the present thesis, as both *Sry* and sex chromosome complement affected subjects' performance differentially according to the tasks used. Research in wildtype rodents has shown that in the light-dark box test of anxiety females display a more anxiolytic pattern of behaviour than males (Hughes, Desmond, & Fisher, 2004; Voikar, et al., 2001), which is consistent with our findings from the FCG model (whereby mice with a XX karyotype spent more time in the aversive area of the light-dark box than mice with a XY karyotype). Furthermore, in this thesis we found that wildtype males exhibited fewer indices of anxiety than wildtype females on the Elevated Zero maze (Chapter IV), consistent with higher *Sry* expression in the former group and with the predictions from the FCG model. These findings in wildtype mice are consistent with some previous literature on sex differences in performance on the elevated mazes (An, et al., 2011; Chiba, et al., 2009), but inconsistent with other studies, whereby wildtype females appear less anxious than wildtype males (Gioiosa, et al., 2007; Hughes, et al., 2004; Rodgers & Dalvi, 1997; Voikar, et al., 2001). The latter studies align with data from our rat knockdown model (whereby reduced *Sry* expression was associated with 'less anxious' behaviour). Clearly the effects of Sry and other sex-linked genes on anxiety-related measures are complex: there may be influenced by strain, by species differences, by the brain sites of *Sry* over/under-expression, or by the specific anxiety-related measures assessed.

In Chapter V we found that transgenic Sry was expressed significantly higher than the endogenous version of the gene. This supra-physiological expression of the *Sry* transgene means that the FCG model could potentially be of more direct utility for modelling the psychological effects of *SRY* over-expression associated with sex-chromosome ploidies (such as 47,XYY) than behavioural variability within the normal range (Mulligan, Gill, & Fitzgerald, 2008; J. L. Ross, Zeger, Kushner, Zinn, & Roeltgen, 2009; Ruud, Arnesen, Larsen Stray, Vildalen, & Vesterhus, 2005).

Data within this thesis have suggested that *Sry* might influence anxiety-related behaviours, activity and feeding behaviours. It is possible that in humans, the gene influences similar domains. Specifically, polymorphisms within the *SRY* gene

affecting protein expression/function might explain the variability in these behaviours, and may help to explain why males are vulnerable to disorders characterised by hyperactivity and protected from disorders associated with anxiety and abnormal feeding behaviours (i.e. eating disorders). However, *SRY* does not display much genetic variability within populations from the same geographical region (E. Stergiakouli, pers. comm.) which may be a barrier to this approach. Furthermore, there are differences in gene structure between *Sry* in rodents and *SRY* in humans (see Chapter I, section 1.3.4). Hence, it is possible that the SRY protein acts to different extents or via different pathways in rodents and humans, which would make any data extrapolation from animal studies difficult. On the other hand, some degree of inter-species similarities in protein function could still be possible; for example, research on hypertension (renin-angiotensin system/RAS) has provided useful information into the action of *Sry* both in humans and in rats (Turner, et al., 2011).

7.5 Future directions

The present thesis has generated a number of findings, which it would be worthwhile pursuing further. A high priority should be to achieve a higher level of *Sry* downregulation in our rat model, possibly with the use of alternative molecular strategies. Small interfering RNAs (siRNAs) have been successfully used in the past to downregulate *Sry in vitro* (Wu, et al., 2009), as well as other genes *in vivo* (Thakker, et al., 2005; Zhang, et al., 2009), and could thus provide another, potentially more reliable and sensitive tool. However, optimising conditions for the successful transfection of siRNAs *in vivo* is difficult (Behlke, 2006). If *Sry* expression can be more successfully downregulated, the resultant effects should be tested in multiple behavioural assays of anxiety and activity. It might be anticipated that more extensive *Sry* knockdown would have concomitant effects on *Th* and *Maoa* (as well as *Fosl1*) downregulation and these might subsequently influence the resultant behaviours.

The effects of *Sry* downregulation on cognitive measures such as habit formation might also be determined, given previous evidence that VTA and *Fosl1* function are involved in this sexually dimorphic process (Jill B. Becker & Hu, 2008; Faure, et al., 2006). Second, whilst our data have provided some evidence that *Sry* downregulation might influence behaviour via *Fosl1* expression, it is likely that *Sry* regulates numerous other brain-expressed genes, which could potentially influence

behaviour. Hence it might be interesting to examine global brain gene expression changes elicited by *in vivo Sry* knockdown with a view to identifying non-obvious genetic targets of *Sry*.

In the FCG model, the SEX CHROMOSOME COMPLEMENT effects observed on food consumption, and on light-dark box measures could be attributed to the influence of X-linked dosage, X-linked imprinting, or Y-linked genetic mechanisms. To clarify which mechanism(s) might be responsible for the present sex chromosome effects, we could test mice with aberrant chromosomal constitutions on the same assays as the FCG mice; such mice might include XO, XXY, and XYY subjects (Arnold, 2009a). A similar strategy has been employed previously to investigate the genetic basis of elevated expression of some genes in XX striatum relative to XY striatum (Chen, et al., 2009). XO mice could be used to test for X-linked gene haploinsufficiency and X-linked imprinting (see Chapter I, section 1.3.7), whereas XXY and XYY mice could provide information on the extent to which overdosage of X or Y chromosomes respectively contribute to the behavioural phenotype under investigation. Work conducted on animal models of Klinefelter syndrome (XXY, and XX^{Y*}) has yielded a role for X chromosome on cognitive phenotypes, as these mice exhibit learning deficits (Lue et al., 2005), and impaired performance in recognition memory (Lewejohann et al., 2009). Furthermore, research on male sexual behaviour has implied that *Sry* might not be the sole gene responsible, as overexpression of Y- chromosome (XYY-) could enhance male sexual behaviour (Park et al., 2008). Together, data from the FCG model and data from models such as these, could illuminate the neural mechanisms underlying sexually dimorphic behaviour in rodents, and could therefore enhance our knowledge about mechanisms underlying sexually dimorphic phenotypes in healthy and clinical human populations.

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Appendix A.

Appendix 1.1. The results of Analysis of Covariance (ANCOVA; OESTROUS STAGE as covariate) on distance travelled ('phenotyper cages') and food consumption.

Behavioural measure	Effect of GENOTYPE	Effect of OESTROUS STAGE
Distance travelled	$F_{1,15}$= .408, p= .532	$F_{1,15}$=1.380, p= .258
Food consumption	$F_{1,15}$=14.683, p< .01*	$F_{1,15}$=1.591, p= .226
*XY-mice consumed more food than XX mice, consistent with results yielded in main analysis.		

Appendix B.

Appendix 2.1. Descriptive data of EPM measures presented on Table 4.3.1i. Data are presented as mean values ± standard error of the mean (SEM).* Analysis was conducted on log10 transformed data.

	Behavioural measure	XX	XX*Sry*	XY-	XY-*Sry*
Total	Distance (cm)*	1084.6±71.3	1031.7±49	1073.2±81.1	1299.3±81.7
	Velocity (cm/s)	3.7± .2	3.2± .1	3.5± .2	3.9± .2
	Rearing*	60.2±6.6	60.5±4.4	55.5±6.8	82.2±8.8
Closed arms	Entries	23.2±2	23.7±2.4	22.6±2.3	26.9±2.4
	Stretch Attends	10.9±1.1	9.2±1.3	10±1.3	9.4±1.3
Open arms	Entries*	12.1±1.4	9±1.5	11±1.5	16.1±2.5
	Head Dips*	2.6± .7	1.5± .8	167± .8	5± .8

Appendix 2.2. Descriptive data of zero maze measures presented on Table 4.3.2i. Data are presented as mean values ± standard error of the mean (SEM).).* Analysis was conducted on log10 transformed data.

	Behavioural measure	XX	XX*Sry*	XY-	XY-*Sry*
Open quadrants	Distance (cm)*	61.2±22.8	124.8±26.5	52.6±25.9	106.4±27.2
	Velocity (cm/s)	3.6± .6	4.8± .7	4.6± .7	4.5± .7
	Head Dips*	1.1± .4	1.7± .5	1.2± .5	1.8± .5

Appendix 2.3. Descriptive data of OF measures presented on Table 4.3.3i. Data are presented as mean values ± standard error of the mean (SEM).).* Analysis was conducted on log10 transformed data.

Zone	Behavioural measure	XX	XX*Sry*	XY-	XY-*Sry*
Total	Distance (cm)	7327.8± 464.5	5723.4± 526.6	6364.4± 526.6	6700.9± 539.6
	Velocity (cm/s)	14.8± .9	12.5±1	11.4±1	13.4±1.1
	Rearing*	125.8±11.9	97.4±13.5	97.1±13.5	137.4±13.8
Outer	Frequency*	34.7±4.4	25.6±4.9	27.8±4.9	35.4±5.1
	Duration*	540.3±7.9	547.6±9	546.1±9	526.8±9.2
Intermediate	Frequency*	46.7±6.2	32.6±7	37.6±7	50.6±7.2
	Duration	46.8±5.9	42±6.7	42.4±6.7	54.1±6.9
Central	Frequency*	12.9±2	8.7±2.3	10.6±2.3	16±2.3
	Duration*	11.9±2.5	10.1±2.8	11.4±2.8	19±2.8

Appendix 2.4. Descriptive data of LD box measures presented on Table 4.3.4i. Data are presented as mean values ± standard error of the mean (SEM).).* Analysis was conducted on log10 transformed data.

Zone	Behavioural measure	XX	XX*Sry*	XY-	XY-*Sry*
Total	Distance (cm)*	1184.5±461.7	1083.4±523.6	1948.4±523.6	1166.2±536.5
	Velocity (cm/s)*	381.5±486.1	485.7±551.2	1438.6±551.2	511.2±564.8
	Rearing	20.5±2.7	16.9±3	11.9±3	16.9±3.1
Dark	Frequency	12.6±1.3	9.6±1.5	8.9±1.5	11.7±1.5
Near	Frequency	27.5±3.2	22.1±3.6	17.3±3.6	22.3±3.7
Far	Frequency	8.3±1.1	5.8±1.2	5.7±1.2	7.4±1.2
	Latency	189.9±31.4	229.7±35.6	204±35.6	191.7±36.4

Appendix 2.5. Descriptive data of foecal boli analysis presented on Table 4.3.5i. Data are presented as mean values ± standard error of the mean (SEM).).* Analysis was conducted on log10 transformed data.

Behavioural task	XX	XX*Sry*	XY-	XY-*Sry*
Elevated Plus Maze*	1.8± .4	1.7± .4	1.6± .3	1.3± .4
Zero Maze*	2.5± .3	2.5± .4	2.1± .5	2.3± .4
Open Field*	4.2± .4	4± .4	4.3± .5	4± .3
Light-Dark Box*	.7± .3	.3± .1	.5± .2	.5± .2

Appendix 2.6 The results of Analysis of Covariance (ANCOVA; OESTROUS STAGE as covariate) on EPM measures.

Arms	Behavioural measure	Effect of GENOTYPE	Effect of OESTROUS STAGE
Total	Rearing	$F_{1,42}$= .029, p= .865	$F_{1,42}$= .001, p= .975
Close	Duration	$F_{1,42}$= .153, p= .698	$F_{1,42}$= .420, p= .520

Appendix 2.7. The results of Analysis of Covariance (ANCOVA; OESTROUS STAGE as covariate) on zero maze measures.

Quadrant	Behavioural measure	Effect of GENOTYPE	Effect of OESTROUS STAGE
Close	Stretch attends	$F_{1,44}$=1.460, p= .233	$F_{1,44}$= .147, p= .703
Open	Entries	$F_{1,44}$=1.618, p= .210	$F_{1,44}$=1.515, p= .225
	Duration	$F_{1,44}$= .185, p= .669	$F_{1,44}$= .004, p= .951
	Rearing	$F_{1,44}$= .927, p= .341	$F_{1,44}$= .331, p= .568

Appendix 2.8. The results of Analysis of Covariance (ANCOVA; OESTROUS STAGE as covariate) on light-dark box measure.

Task	Behavioural measure	Effect of GENOTYPE	Effect of OESTROUS STAGE
Light-dark box (near zone)	Latency	$F_{1,45}$= .432, p= .514	$F_{1,45}$= .001, p= .981

Appendix C.

Appendix 3.1. The results from weight measurement and consummatory behavioural analysis between sense and acsf group

	Behavioural measure	Sense (n=8)	Acsf (n=6)
Pre-operation	Body weight *	340±9.4	344.4±9
	Drinking pattern (ml per day)†	21.6± .8	19.7±1.1
Post-operation	Body weight *	360.6±8.6	366.8±9.8
	Drinking pattern (ml per day)†	21.5± .9	22.9±1.4

* **repeated measures ANOVA**; main effect of TIME ($F_{1,12}$ = 140.601, p<.01). No main effect of GROUP ($F_{1,12}$ = .162, p= .694) or interaction between TIME*GROUP ($F_{1,12}$ = .246, p= .629). † **repeated measures ANOVA**; no main effect of TIME ($F_{1,12}$ = 1.670, p= .221.) or GROUP ($F_{1,12}$ = .055, p= .819) ; no interaction between TIME*GROUP ($F_{1,12}$ = 2.069, p= .176).

Appendix 3.2. The results from LMA analysis between sense and acsf group

Behavioural Measure	Sense (n=8)	Acsf (n=6)
Beam breaks, Day 0 *	629±57	610.1±52.5
Beam breaks, Day 1 *	618±42.3	631.8±57.4
Beam breaks, Day 2 *	413.9±33.5	556±86.9
Beam breaks, Day 3 *	466.1±43.3	384.8±49.1
Beam breaks, D-Amphetamine †	474.5±48.5	768.3±266.8

* **repeated measures ANOVA**; no main effect of DAY ($F_{2,22}$ = 0.819, p= .454.) or GROUP ($F_{1,11}$ = .574, p= .464); no interaction between DAY*GROUP ($F_{2,22}$ = 2.254, p= .129) or DAY*BASELINE ACTIVITY ($F_{2,22}$ = 0.371, p= .695). † **independent t-test**; $t_{5.331}$ = -1.083, p= .325

Appendix 3.3.The results from Balance beam analysis between sense and acsf group

Behavioural measure	Sense (n=8)	Acsf (n=6)	Effect of GROUP
Crossing time (s)	6± .6	6.5±1.3	t_{12} = -.397, p= .698
Falls	0.12± .1	0.3±0.2	U=19, p= .538
Slips	0	0	n/a

Appendix 3.4.The results from EPM analysis between sense and acsf group

	Behavioural Measure	Sense (n=8)	Acsf (n=6)	Effect of GROUP
Total	Distance (cm)	1586.9±130.3	1704.5±128.5	t_{12} = -.627, p= .543
	Velocity (cm/s)	5.3±.4	5.4±.4	t_{12} = -.092, p= .929
	Rearing	36.5±5.1	38.2±2.8	t_{12} = -.261, p= .798
Closed Arms	Entries	12.5±2.4	11.2±1.5	t_{12} = .434, p= .672
	Time (s)	99.6±19.3	120.6±21.9	t_{12} = -.717, p= .487
	Stretch Attends	7.2±.8	5.8±.7	t_{12} = 1.233, p= .241
Open Arms	Entries	15.1±2.8	13.8±1.8	t_{12} = .353, p= .730
	Time (s)	141.4±22.0	125.6±20.6	t_{12} = .507, p= .621
	Head Dips	15.6±1.8	10.6±2.1	t_{12} = 1.815, p= .095

Appendix 3.5. The results of gene expression analysis between sense and acsf group

	Effect of GROUP
Sry	$t_{10.997}$ = -.062, p= .951
Th	t_{11} = .619, p= .548
Maoa	t_{11} = .793, p= .444
Tph *	U=9, p= .202
*Fosl1**†	t_9 = -1.575, p= .150
n=7 for sense, n= 6 for acsf. * n=5 for acsf. †n=6 for sense.	

Appendix 3.6. The results of brain-damage related gene analysis between sense and acsf group

	Effect of GROUP
Sst	t_{10} = .-1.571, p= .147
Plp	t_{10} = -.128, p= .901
Gat1	$t_{6.203}$ = -.063, p= .951
n=7 for sense, n=5 for acsf.	