A NOVEL MOUSE MODEL FOR PARTIAL ANDROGEN INSENSITIVITY SYNDROME

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by

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Abstract

Androgen Insensitivity Syndrome (AIS) is the under-masculinization of individuals with XY sex chromosome karyotypes. A broad clinical spectrum of AIS exists, from mild to partial to complete AIS. Mouse models of complete AIS have been used to study aspects of sexual development, physiology, and behavioural outcomes in the absence of androgenic signaling. There is currently no animal model of partial AIS (PAIS), and the novel mouse strain described in this research satisfies the clinical description of human PAIS patients, appearing outwardly male with additional feminine characteristics. My research on the PAIS mouse model focuses on the anatomical features and endocrinology of this unique strain, and the role of partial androgen signaling as a cause of behavioural anxiety.

Anatomically, PAIS male mice have similar body size and weight to wild-type (WT) males, but they have an intermediate anal-genital distance that is shorter than WT males, but longer than WT female mice. The PAIS males do possess intra-abdominal testes, but they are infertile, as they lack internal reproductive structures such as the seminal vesicles, prostate, epididymis and vas deferens. Androgen-responsive organs are significantly smaller in mature PAIS males compared to age-matched WT males, including the testes, preputial glands and kidneys; however, this phenotype did not correlate with a lack of testosterone (T) synthesis, since T concentrations were not different between WT and PAIS males as juveniles (30 d) or young adults (50 d). Following an androgen sensitivity test, androgen-responsive growth of the preputial glands in castrated males was significantly reduced in T-supplemented PAIS versus WT

males, indicating partial androgen insensitivity. A defect in androgen sensitivity was further indicated by the elevated serum gonadotropin concentrations at 30 d of age (follicle stimulating hormone (FSH)) and 50 d of age (FSH and luteinizing hormone (LH)), suggesting a failure of negative feedback regulation at the level of the hypothalamic-anterior pituitary-gonadal (HPG) axis. Behaviourally, the PAIS mutation significantly decreased male-typical behaviours (aggression and sexual interest) and increased anxiety-like behaviour in a standard paradigm for measurement of rodent anxiety – the elevated plus maze. One assay of social behaviour showed no difference in social interactions between PAIS and WT male mice. This novel rodent model of PAIS satisfies the criteria for human PAIS patients, and will serve as an excellent tool to further explore the potential consequences of PAIS to male health.

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List of Abbreviations

µl	Microliter
µg	Microgram
A	Adult
AIS	Androgen insensitivity syndrome
AMH	Anti-Müllerian hormone
AR	Human androgen receptor protein
AR	Human androgen receptor gene
Ar	Rodent androgen receptor protein
Ar	Rodent androgen receptor gene
ARKO	Androgen receptor knockout
BNST	Bed nucleus of the stria terminalis
BSTpm	Posteromedial Nucleus of the Bed Nucleus of the Stria Terminalis
C	Celsius
CAH	Congenital adrenal hyperplasia
CAIS	Complete androgen insensitivity syndrome
Chr	Chromosome
cm	Centimeters
cm/s	Centimerters per second
d	Day
DHT	Dihydrotestosterone

DNA	Deoxyribonucleic acid
EPM	Elevated plus maze
FSH	Follicle stimulating hormone
g	Grams
GABA	Gamma-aminobutyric acid
GD	Gonadal dysgenesis
GnRH	Gonadotropin-releasing hormone
HCL	Hydrochloric acid
HPG	Hypothalamic-pituitary-gonadal
h	Hour
J	Juvenile
kg	Kilograms
LC	Locus coeruleus
LDB	Light/dark box
LH	Luteinizing hormone
n	Sample size
NaOH	Sodium hydroxide
ng/dl	Nanograms per deciliter
ng/ml	Nanograms per milliliter
M	Molar
MAIS	Mild androgen insensitivity syndrome
MePD	Posterodorsal medial amygdala
mg	Milligrams

min	Minute
mm	Millimeters
OF	Open field
OMIM	Online Mendelian Inheritance in Man
PAIS	Partial androgen insensitivity syndrome
PCR	Polymerase chain reaction
rpm	Revolutions per minute
s	Seconds
SCN	Suprachiasmatic nucleus
SI-OF	Social interaction open field
SNB	Spinal nucleus of the bulbocavernosus
SRY	Sex-determining region Y
TBE	1 x TRIS-Borate-EDTA
TDF	Testis determining factor
T	Testosterone
Tfm	Testicular feminization mutation
VMH	Ventromedial hypothalamus
V	Volts
wk(s)	Week(s)
WT	Wildtype

Chapter 1 - INTRODUCTION

1.1 Androgens and Male Reproductive Health

1.1.1 Normal Male Development

Phenotypic sex differences are attributed to the sex chromosomes (Chr), X/X in females and X/Y in males. The developmental path starts from a single-celled zygote that develops to form an embryo and later progresses into a fetus. The zygote forms when the female gamete (egg) is penetrated with the male gamete (sperm). Genetic materials are exchanged between egg and sperm resulting in a zygote. The zygote undergoes multiple cell divisions to form a large cell mass termed the morula and subsequently a blastocyst (Harrison, 1959). The blastocyst implants into the uterine wall and continues to grow into an embryo (Harrison, 1959). The embryo undergoes sexual differentiation mediated by the presence of Chr X and Y (Harrison, 1959). Generally, the Chr XY pairing leads to the development of male sex organs and the Chr XX pairing results in the development of female sex organs (Arnold, 2004). Studies on human subjects whose phenotypic sex did not correlate with their genotypic sex led to the isolation of the *SRY* (*Sex-determining Region Y* gene). The *SRY* protein is necessary for male development and is often referred to as the Testis-determining Factor (TDF) (Griffen, 2000) (Fig 1.1). The expression of SRY protein from the male Chr Y leads to the differentiation of the embryonic gonads into testes in both humans and rodents (Sinclair et al., 1990).

1.1.2 Hormonal Influences and Male Development

Male development continues with the hormonal contribution of the gonad. Gonadal hormones further sexual differentiation by contributing to the sex-specific

development of many organs and have permanent organizational effects on the internal reproductive anatomy. During gestation, the embryo develops two sets of ducts which are the undifferentiated precursors for much of the male and female reproductive tracts; both the Wolffian and Müllerian ducts normally develop in the presence of Chr Y (Griffen, 2000). Mullerian ducts in females give rise to the fallopian tubes, upper vagina, and the uterus; in males, the Wolffian ducts give rise to the seminal vesicles, vas deferens, and epididymis (Fig 1.2) (Griffen, 2000). A variety of hormones are required for these structures to develop normally. Androgens are steroid hormones that in large part regulate development and maintenance of male sexual characteristics in vertebrates (Siiteri & Wilson, 1974). Male reproductive sex organs respond to androgens and do not form in their absence, with the exception of the testis. Testosterone (T) (Fig 1.3) is necessary for the appearance of the male phenotype and is secreted by the interstitial cells of Leydig of the testes (Brinkmann, 2001). Anti-Müllerian Hormone (AMH) from the fetal testes causes the regression of Müllerian ducts (Griffen, 2000). External masculine genitalia including the phallus and scrotum begin to develop following a surge of T secretion from newly formed Leydig cells. The testes descend shortly after development (Corbier et al., 1992).

Male secondary sex characteristics developing at puberty are also androgen dependent (Parker, 2004). At puberty, males undergo physical changes such as skin acne, growth of hair on the axilla, face, chest, and abdomen, maturation of the body, increases in muscle and bone mass, deepening of the voice, and continued growth of the penis and testes; these changes are due to a surge in androgenic hormones (Goldstein & Wilson,

1975; Parker, 2004). Thus normal male sexual differentiation and pubertal maturation are dependent upon gonadal androgen secretion.

Studies using genetically engineered *AR* knock out mice (ARKO) demonstrated T's role in masculinization. Deletion of *Ar* from all tissues of XY mice resulted in a feminized external appearance (genitalia), eventual testis atrophy and a decline of T production (Notini, 2005).

1.1.3 Signaling Mechanisms

Effects of androgens may be mediated by a signal transduction mechanism which requires testosterone binding to the androgen receptor (AR) protein (Brinkmann, 2001); the AR protein is a member of the steroid binding nuclear receptor superfamily and has an affinity for binding to dihydrotestosterone (DHT) or T leading to transcriptional control of downstream genes (MacLean et al., 1997b; Heemers & Tindall, 2007). DHT is a potent metabolite of T, which can bind to AR with higher affinity than T and has longer lasting effects; T is converted to DHT via the enzyme 5-alpha-reductase (Fig 1.3) (Wilbert et al., 1983; MacLean et al., 1997b). DHT and its action is important for the development and maintenance of male secondary characteristics such as pubertal hair, masculinization of the penis and scrotum, and formation of the prostate (Jost, 1972; Vague, 1983; Warne & Kanumakala, 2002).

The *AR* gene is located in humans at Xq11-12; therefore, females have two copies of the *AR* gene and males have one copy. Reduced function or complete loss of function due to mutations of the *AR* gene interferes with AR signaling. This altered signaling can give rise to various disorders of sexual development. As males have only one copy of the *AR* gene, these genetic abnormalities can be readily observed in males.

Androgens also have the ability to exert their effects directly on cell surface receptor of target cells without AR binding and nuclear translocation; these responses are faster and work through such systems as the gamma-aminobutyric acid (GABA) receptor or the sex hormone binding globulin receptor (Walker, 2003; Foradori et al., 2008). Although androgens may act through different mechanisms, the AR receptor is the predominant pathway for sexual differentiation.

1.1.4 Hypothalamic-Pituitary-Gonadal Axis

The hypothalamic-pituitary-gonadal (HPG) axis is critical for male development and regulation of the reproductive system. Within the HPG axis, the hypothalamus produces gonadotropin-releasing hormone (GnRH), the anterior lobe of the pituitary gland produces follicle stimulating hormone (FSH) and luteinizing hormone (LH), and the gonads produce both estrogens and androgens. GnRH is secreted from the cells of the hypothalamus and is carried to the anterior pituitary via the hypophyseal portal venous system. In response to GnRH, the pituitary produces and releases LH and FSH into the blood stream (Griffen, 2000). Both LH and FSH affect the production of gonadal hormones but their roles differ in males and females. The role of LH in males is to stimulate interstitial cells in the testes to increase T production, while FSH plays a critical role in spermatogenesis.

In humans, serum androgen levels peak at puberty and average levels remain constant as young men transition into adulthood. Decreases in HPG activity are observed during aging as are decreased levels of T (Heller, 1944). This decrease in T levels can lead to age-related hypogonadism and can result in a loss of muscle mass, an increase in

visceral fat stores, a decline in sexual drive (libido), impotence, a decreased attention span, and an increased chance of bone fracture (Heller, 1944; Vermeulen, 1979). The collective symptoms referred to as andropause, or male menopause, increases in prevalence from 7% in middle aged men to 20% among men ages 60 through 80 (Vermeulen, 1979).

1.1.5 Other Effects of Androgens

Testosterone exerts effects on the male reproductive pathway, but also affects secondary sex characteristics and sexual dimorphisms in the brain and body. Early rodent castration studies showed the importance of androgens and effects of early T deprivation to male physiology and behavioural outcomes. Recognition that males were generally more sensitive to androgenic deprivation than females led to the modern concept of gender-specific health research.

Sexual dimorphisms in behaviour mediated by gonadal hormones exist in sexually reproducing animals, (Phoenix et al., 1959; Jost, 1972; Juntti et al., 2008). Early exposure to the organizational effects of T masculinizes the developing brain leading to enduring behavioural changes in a variety of animal models (Morris et al., 2004). These behaviours are mediated by permanent organizational effects on the brain. AR receptors are expressed in various brain regions implicated in male typical mating behaviours such as the bed nucleus of the stria terminalis and the hypothalamus (Shah et al., 2004). T exposure during early critical developmental periods produces permanent behavioural changes in aspects such as child play behaviour, sexual orientation, and gender identity (Hines & Kaufman, 1994).

Sexual dimorphisms may be anatomical (color, size, coat pattern, organ

arrangement), physiological, or behavioural differences between sexes of the same species. Several brain structures, which are sensitive to AR signaling, are sexually dimorphic. The ventromedial hypothalamus (VMH), is a sexually dimorphic area involved in sexual behaviour that contains a significantly higher concentration of Ar in males compared to females (Morris et al., 2004). The *Ar* gene appears to play a role in the masculinization of the VMH; male rats with a defective *Ar* gene appear to have no difference in their VMH when compared with females (Morris et al., 2004). The bed nucleus of the stria terminalis (BNST) is also sexually dimorphic; a region within this area, the posteromedial nucleus of the bed nucleus of the stria terminalis (BSTpm), contains greater Ar density and has a larger volume in male compared to female rats (Madeira et al., 2001). Normal male rats and *Ar* mutated rats differ in BSTpm volume (Roselli, 1991) indicating a role for Ar in brain masculinization. Finally, wildtype mice have significantly smaller locus coeruleus (LC) volume and neuron population compared to males with Ar defects (Garcia-Falgueras et al., 2005). The LC is heavily implicated in the stress response and Ar signaling masculinizes this region (Garcia-Falgueras et al., 2005). Female mice have a larger LC, therefore the masculine phenotype is a reduced LC volume (Garcia-Falgueras et al., 2005).

Spontaneous mutant and genetically engineered mouse models show that *Ar* function affects behaviour. Male mice bearing a spontaneous null allele of the *Ar* gene not only look feminine in their external appearance, but they fail to display behaviours appropriate to their sex (mounting, patterns of aggression) (Lyon & Hawkes, 1970). *Ar* knockouts (ARKO) are genetically manipulated mice in which the *Ar* gene is completely turned off. ARKO mice have impaired male-typical behaviours including sexual interest

(mounting, intromissions) and aggression (Sato et al., 2004). Similar studies of male-typical behaviours show that castration of male mice abolishes aggressive behaviours that can be restored by administering androgens (T and DHT) (Gandelman, 1980). *Ar* defective mice, with intact testes, still show decreased aggressive behaviour suggesting a role for androgens in controlling this specific behaviour (Ohno et al., 1971). Similar to the ARKO mice, mice with severe *Ar* genetic mutations have impaired male sexual behaviours (Ohno et al., 1971). Mice lacking selective Ar function in the entire nervous system show similar behavioural deficits in male-typical behaviours. These mice show lower sexual motivation, intromissions, and reduced sperm count, and also display low aggressive behaviours during the resident-intruder test of aggression with an unfamiliar male (Raskin et al., 2009). Males lacking Ar also displayed significantly fewer mounts and intromissions towards females, as well as less time spent fighting and fewer attacks in aggression tests (Juntti et al., 2010). Knowledge that androgens affect behaviour furthers the understanding of gender typical reactions in various behavioural situations, mediated by organizing effects on the brain.

Figure 1.1: Gonadal differentiation determined by presence or absence of TDF

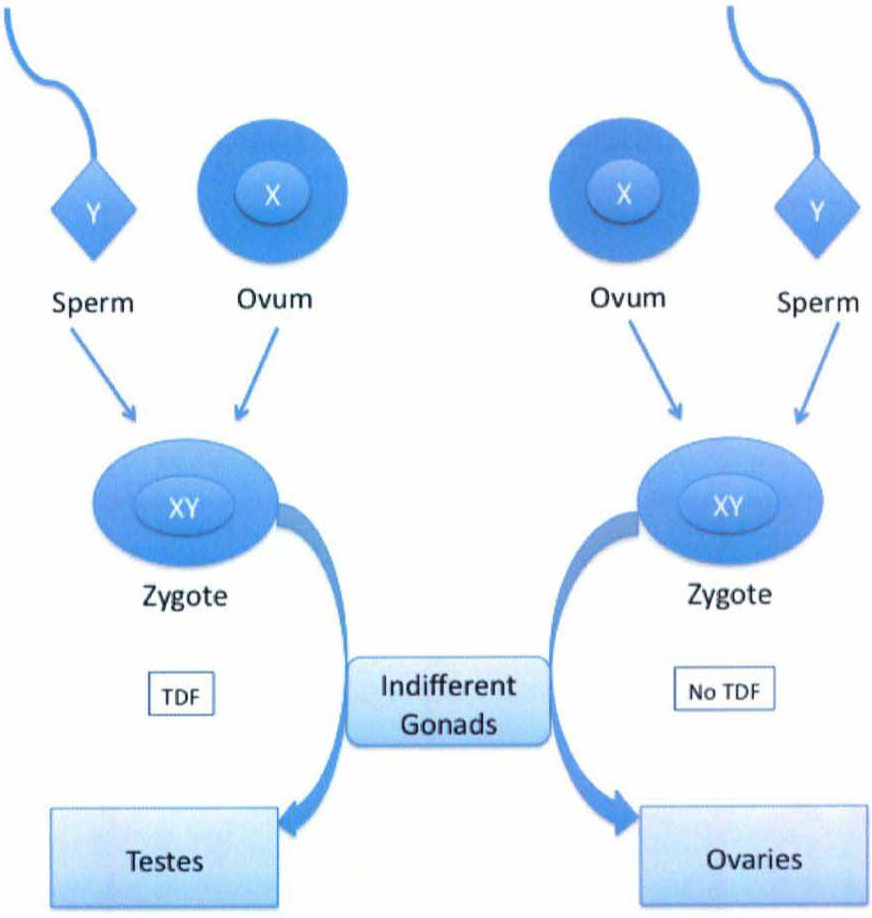


Figure 1.1 shows the pathway of gonadal differentiation initiated by the presence or absence of testis determining factor (TDF).

Figure 1.2: Bipotential gonad can develop into either a female or male gonad

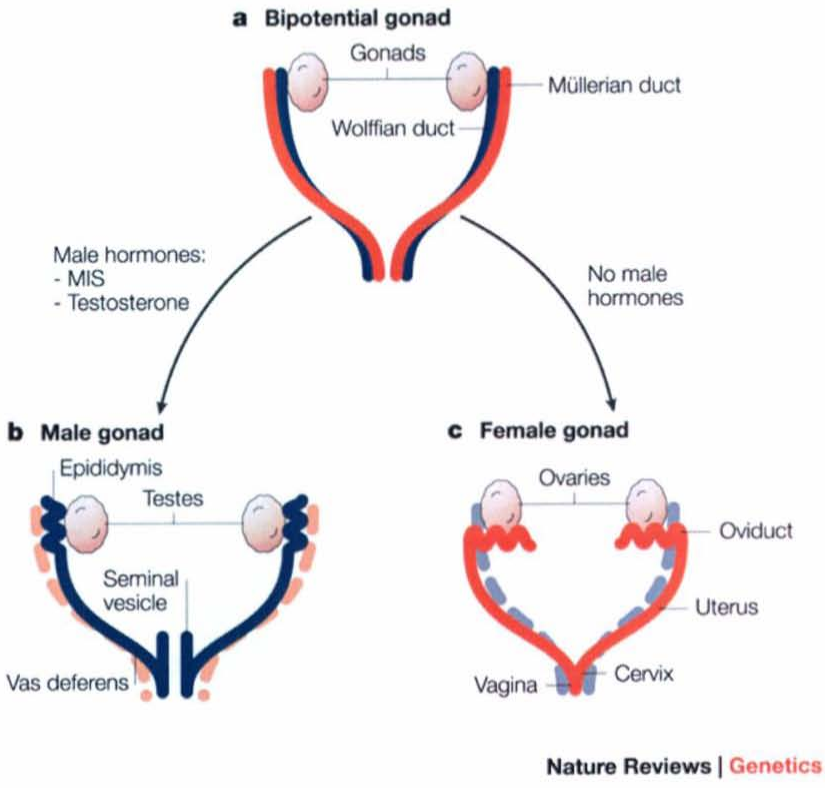


Figure 1.2 shows the bipotential gonad differentiating into the male or female gonad structures under the influence or absence of male hormones. Permission of figure use was granted from Nature Reviews (Appendix A) (Kobayashi & Behringer, 2003).

Figure 1.3: Chemical structures of testosterone (T) and dihydrotestosterone (DHT)

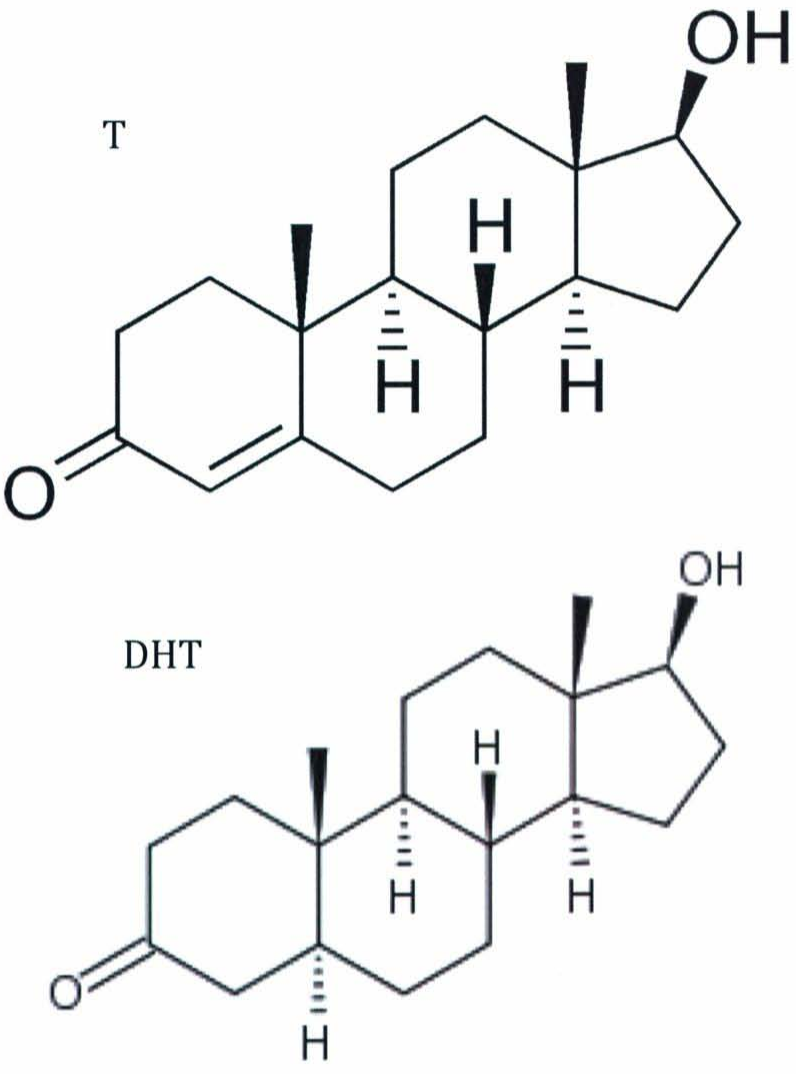


Figure 1.3 shows the chemical structures of androgens T and DHT. T is converted into DHT by interactions with the enzyme 5-alpha-reductase.

1.2 Disorders of Human Sexual Development

Disorders of sexual development can originate at the level of the chromosome, the gonad, or the downstream response to gonadal hormones. Disorders of chromosomal sex include Klinefelter's Syndrome and Turner's Syndrome. Individuals with Klinefelter's Syndrome are born with a 47 XXY Chr karyotype and look phenotypically male but have demasculinized features such as small testes, decreased sperm count, low to normal serum T levels but elevated estradiol and FSH levels (Parker, 2004). Turner's Syndrome manifests in phenotypic females; these females lack an entire X chromosome (45 XO Chr karyotype). Characteristics of Turner's Syndrome include lack of menses and feminine secondary sex characteristics (Parker, 2004).

Some gonadal disorders are found despite a normal karyotype and are due to an over or under production of androgens. Patients with pure gonadal dysgenesis have an immature female phenotype although they have normal 46 XX or 46 XY karyotypes (Parker, 2004; Sharma & Gupta, 2008). Spectrums of undervirilized to completely overvirilized 46 XY individuals have also been described. These individuals have no Müllerian tissue present, thus their disruption in androgen production or overproduction occurred after AMH was secreted (Parker, 2004).

Phenotypic sex disorders include female pseudohermaphroditism, male pseudohermaphroditism, and true hermaphroditism (ovary & testis) (Johannsen et al., 2006). In this subset of disorders, the phenotypic sex of the individual disagrees with their gonadal sex (Parker, 2004). Sufficient levels of androgens do not guarantee their proper functioning. Many *AR* mutations do not allow androgens to bind properly to the receptor and therefore have significant effects on aspects of behaviour, physiology and anatomy.

The production of T by the fetal testis is essential for normal male masculinization, and it has been demonstrated that the fetal testis can produce T from 8-21 weeks of development (Siiteri & Wilson, 1974). Treatment of gender disorders may include appropriate surgical procedures, hormonal treatments, and various forms of psychological support systems. Furthermore, treatment of these disorders must be individualized based on such factors as age at presentation, economic status and psychosocial issues (Sharma & Gupta, 2008).

The quality of life of those living with disorders of sexual development has been studied (Johannsen et al., 2006). Results of such a study are used to determine appropriate treatments and impacts of medical intervention on function and social well-being. An impaired quality of life and more affective stress relative to control subjects was observed in individuals with complete AIS (CAIS), 46XX or 46XY karyotypes with some degree of virilization, 46 XY gonadal dysgenesis (GD), or congenital adrenal hyperplasia (CAH) which is an autosomal recessive disorder. Virilized XX and under-virilized XY patients were significantly more likely to have lower incomes, and spousal relationships were significantly less frequent in CAIS and CAH patients (Johannsen et al., 2006). All patients were significantly less likely to have children, and had a tendency towards a higher frequency of counseling due to severe psychological symptoms (such as suicidal thoughts) and increased anxiety levels (Johannsen et al., 2006).

Our knowledge of human sexual development is advanced by the study of gender disorders such as CAH. In females it causes virilization of the external genitalia and atypical female behavioural patterns. Young girls with CAH have an increased male-typical play pattern (preference for male toys, male companions, and rough and tumble

play) (Hines & Kaufman, 1994). These findings support the idea that T influences psychosexual development outcomes that are evident in individuals with complete androgen insensitivity syndrome (CAIS).

1.3 Androgen Insensitivity Syndrome

Androgen Insensitivity Syndrome (AIS) is a well-characterized disorder of male sexual differentiation, first described in 1953 by John Morris after reviewing 82 cases (Morris, 1953). This rare genetic disease affects 46 XY individuals with appropriate amounts of serum T for normal males (Morris, 1953) and results in varying degrees of under masculinization based on the degree of function present in the AR protein (MacLean et al., 1997a). The Online Mendelian Inheritance in Man number (OMIM #) for AIS is (OMIM #300068). Three phenotypes of AIS exist within the AIS spectrum and are diagnosed as separate clinical subgroups: mild AIS (MAIS), partial AIS (PAIS), and complete AIS (CAIS) (Quigley et al., 1995). The prevalence of CAIS is estimated to be about 1:20,000 male births and although the prevalence of PAIS is unknown, it is assumed to be almost equal to that of CAIS (Quigley et al., 1995). MAIS is likely to be more common, with approximately 10% of phenotypically normal men with unexplained infertility having some mild degree of androgen insensitivity (Schulster et al., 1983). The genetic defect is carried on Chr X, and although inherited as an X-linked recessive trait, approximately 30% of cases are anticipated to be sporadic mutations (Hughes & Deeb, 2006). AIS is the most common identifiable cause of male pseudohermaphroditism (Ahmed & Hughes, 2002). Critical periods exist for the consequence of AIS in humans during the first surge of T synthesis in the embryo (3.1cm of development) (Siiteri & Wilson, 1974). Based on clinical findings, the diagnosis of AIS must follow

determination of hormonal levels in order to rule out potential deficiencies in androgen production.

1.3.1 Mild Androgen Insensitivity Syndrome (MAIS)

MAIS is characterized by impaired spermatogenesis and underdeveloped genitalia. It may also explain cases of impaired spermatogenesis despite a typical male physique (Vague, 1983; Quigley et al., 1995; Brinkmann, 2001). Unlike the other phenotypes, MAIS may go completely unnoticed, as some of its characteristics overlap with those of normal men harboring no *AR* mutation or defect. A report on the frequency of MAIS in a population of phenotypically normal men was conducted (Aiman et al., 1979). Men were taken from a clinical population suffering from severe oligospermia (reduced sperm count), and azoospermia (no detectable sperm count) (Aiman et al., 1979). An AIS insensitivity index was devised to identify individuals with AIS and determine its frequency, The AIS index exploits the relatively elevated levels of LH and T found in these individuals (Aiman et al., 1979). Aimen and colleagues concluded that a score >200 on the AIS index indicated MAIS; normal males with normal sperm concentrations had a mean index score of 102. In a similar retrospective study, 86 patients suffering from either azoospermia or oligospermia were included, and, of this sample, 11.6% had scores placing them in the defined range for MAIS (Schulster et al., 1983). They concluded that the AIS index identified a group of severe oligospermic and azoospermic patients with AIS but that more cases of MAIS are not detectable by this method because they are less severe, and sub grouped differently within the infertile population (Schulster et al., 1983). Thus, the true prevalence of MAIS is not easily calculated.

1.3.2 Partial Androgen Insensitivity (PAIS)

PAIS individuals exist in the middle of the clinical AIS spectrum and have the most varied presentation. The phenotype can range from a predominantly female appearance, to a person with ambiguous genitalia, or a predominantly male appearance with some feminine characteristics (Reifenstein Syndrome) (Vague, 1953; Quigley et al., 1995). Reifenstein Syndrome is the human PAIS condition of male pseudohermaphroditism, which results in infertility and sexual dysfunction. Due to the wide range of clinical presentations in PAIS patients, it is often difficult to measure the actual prevalence of this subtype of AIS; the OMIM number for PAIS is (OMIM #312300). PAIS patients with a predominantly female phenotype often have such features as a fused labia, some pubic hair at the time of puberty, and a small enlargement of the clitoris, whereas those having a more male external phenotype often have a small penis and more body hair (Ferlin et al., 2006). Both groups have undescended testes and elevated levels of LH and T relative to unaffected males (Brinkmann, 2001). More severe *AR* mutations lead not only to sexual development disorders, but also physiological changes in height and bone density. The anatomy of individuals with PAIS is intermediate between those predicted for males and females. Decreased bone density in the lumbar spine is also common (Danilovic et al., 2007).

1.3.3 Complete Androgen Insensitivity Syndrome (CAIS)

In CAIS, the AR protein is completely non-functional or absent and thus individuals appear predominantly female at birth. People with CAIS cannot respond to androgens and do not have a typical masculine appearance, nor do they exhibit masculine behaviour patterns despite the presence of a Y chromosome. They are missing the

Wolffian- derived structures such as the vas deferens, seminal vesicles, prostate and epididymis (Vague, 1983; Quigley et al., 1995). True hermaphrodites possess both ovarian and testicular tissue; however, CAIS individuals are male pseudohermaphrodites, as they have only male gonads present but with female external characteristics (Morris, 1953). Peripheral tissues are completely unresponsive to T, and there is no development of the secondary sex characteristics (Morris, 1953). John Morris first described adult CAIS patients as having the following characteristics: female body type with normal feminine fat deposits, development of normal female breasts, absent or reduced axillary and pubic hair, female external genitals, absent female internal genitals with the exception of some rudimentary glands, small undescended testes, and elevated gonadotropins in some instances (Morris, 1953). Rarely do those with CAIS have any Müllerian-derived internal genitalia (Dodge et al., 1985; Swanson & Coronel, 1993). Based on genotype-phenotype analyses, it was concluded that no real phenotypic variation in CAIS patients exist, however it is frequently observed in PAIS males (Boehmer et al., 2001). Similar to PAIS patients, CAIS patients are intermediate in height between normal XY males and XX females, and they also had a lower than normal bone density in the lumbar spine (Danilovic et al., 2007).

CAIS males differed from normal males in serum levels of LH, FSH and T concentrations and thus are endocrinologically similar to the hormonal profile of PAIS patients. One study compared 10 subjects (XY males) with CAIS; these patients had a typical female appearance, genitalia, and breast development (Amrhein et al., 1976). CAIS males had normal to slightly elevated serum FSH levels, significantly higher serum LH levels, and T and DHT levels within or above the normal range for adult males

(Amrhein et al., 1976). As LH levels are elevated, this indicates a problem with androgen resistance at the level of the HPG axis. This indicated that although there is a problem in androgen signaling, androgen synthesis is not impaired, as shown by the direct T and DHT assays.

The typical presentation of a CAIS patient is primary ammenorhea during "female" puberty. CAIS patients tend to self-identify as female and assume their core gender identity as the sex of rearing, which is usually female (Morris, 1953) (Wisniewski et al., 2000). CAIS diagnosis is missed at birth as the external appearance is female, and karyotyping is not a routine newborn test and is arguably not warranted, as the condition is rare; approximately 1:20,000 male births (Quigley et al., 1995). CAIS diagnosis at adolescence is devastating, and psychological studies have addressed the impact; relative to normal males, CAIS patients have a lower socioeconomic status and a higher tendency towards psychiatric counseling often due to suicidal thoughts and tendencies (Morris, 1953; Johannsen et al., 2006). Using the Hopkins Symptom Checklist (SCL-90-R), CAIS patients also reported higher anxiety levels (Johannsen et al., 2006). Based on levels of mental distress, poor social status, and high levels of anxiety, CAIS males appear to have an impaired quality of life. Another study, however, has not confirmed these findings (Hines et al., 2003). Hines et al. (2003) conclude that CAIS patients are similar to well adjusted females. It is hard to categorize humans due to the vast differences in social experiences and personalities. Perhaps more specific studies that could adjust for social experience may help reconcile the differences between quality of life scores across these reports.

Rodent models of CAIS have been described extensively (Section 1.4). In general, the CAIS males have feminine secondary sex characteristics and are infertile (Quigley et al., 1995). Animal models of AIS have been very useful to broaden our knowledge of this gender disorder given the rarity of the disease in the human population, and the ethical issues that arise with human investigations.

1.4. Animal Models of AIS

1.4.1 *Tfm* in Mice

Lyon and Hawkes (1970) were first to report a Chr X-linked gene for CAIS in a mouse, historically named the *Testicular feminization mutation* (*Tfm*). CAIS Chr XY male mice cannot be outwardly distinguished from Chr XX females due to their feminized external genitalia, although they possess bilateral testes within the abdomen (Lyon & Hawkes, 1970; Ono et al., 1974). The *Tfm* mutation results from a single base deletion of the *Ar* gene causing a frameshift mutation and consequently non-functional Ar protein (Charest et al., 1991). Mouse models of CAIS arising from either spontaneous mutations of the *Ar* gene (such as *Tfm*) or genetically engineered deletions of this gene (knockouts), in laboratory strains of mice result in the same phenotype of a feminized Chr XY male (Hutson, 1986; Charest et al., 1991). Migeon and colleagues (1981) demonstrated the homology of the locus in human CAIS to that observed in the CAIS mouse, and many AR gene mutations are currently catalogued as causative for human AIS.

One of the most important conclusions derived from the *Tfm* mutant mouse was that the *Ar* gene does not control primary sex determination at the level of the gonad, since the testes are fully differentiated in *Tfm* mice. As previously discussed, this process

is attributed to signaling events initiated by the *Sry* gene on Chr Y. Another important conclusion derived from the *Tfm* mutant was that the Ar signaling cascade controls the downstream events required for normal differentiation of the male reproductive tract in mammals (Morris, 1953; Lyon & Hawkes, 1970). In human and rodent species, loss of function mutations in the *AR* (*Ar*) gene can lead to resistance to androgen action and pseudohermaphroditism as the primary phenotype.

Given the ubiquitous role of androgen signalling in male development and physiology, spontaneous and genetically engineered models of AIS in rodents have been instrumental in determining the post-natal health outcomes of AIS. Endocrinologically, *Tfm* males have significantly reduced serum T when compared to WT siblings at adulthood (Table 1.1) (Jones et al., 2003). Low serum T levels are due to a deficiency in the 17α hydroxylase enzyme. The low T level is thought to occur because the testes are located intra-abdominally and in part by loss of Ar function (Murphy & O'Shaughnessy, 1991). However, evidence suggests their levels are similar during the perinatal period (Goldstein & Wilson, 1972) with levels being comparable to normal male mice up until the neonatal stage (1-10 days post birth) of development (Goldstein & Wilson, 1972). Regardless of the T levels, androgen insensitivity leads to elevated serum gonadotropin levels (FSH/LH) in juvenile and adult *Tfm* males relative to age-matched WT males, since negative feedback regulation of the HPG axis is disrupted (Lyon & Hawkes, 1970; Goldstein & Wilson, 1972). FSH and LH are gonadotropins released from the anterior pituitary; they stimulate the gonads to produce sex steroids, such as androgens. In the case of *Tfm* in rodents, both LH and FSH levels are significantly increased and due to the

decreased levels of T in the system there is no signal for negative feedback for the production of FSH and LH.

Tfm rodent models have been enormously helpful to reveal contributions made by Ar-mediated signalling to sexual dimorphisms in the brain. Many anatomic differences have been found in male mice with the *Tfm* mutation relative to WT males; both neuronal soma size and regional volume are significantly decreased in *Tfm* males within the spinal nucleus of the bulbocavernosus (SNB), posterodorsal medial amygdala (MePD), ventromedial hypothalamus (VMH), and the suprachiasmatic nucleus (SCN) (Breedlove & Arnold, 1980; Madeira et al., 2001; Durazzo et al., 2007; Zuloaga et al., 2008). *Tfm* mice have also been extensively studied behaviourally; CAIS mice consistently show a lack of male-typical behaviours (Table 1.2). One aspect of male typical behaviour is their sexual interest towards female mice; when presented with females in estrus, CAIS mice exhibited virtually no mounting, intromissions, or any type of typical male sexual behaviour (Ono et al., 1974). This decline in male sexual function did not correlate with an increase in female sexual behaviour (Ono et al., 1974). Another male-typical behaviour is aggression in the form of chasing, biting, fighting and wrestling. During a standard test of aggression, the resident-intruder test, *Tfm* mutants were less aggressive towards male intruders in their homecages than WT males (Ono et al., 1974). *Tfm* male mice were also used to assess anxiety-like behaviour. *Tfm* males showed increased anxiety on the novel object test as well as the Light/Dark Box (LDB) compared to WT males (Zuloaga et al., 2008). Overall, congenital defects in Ar signalling in the mouse have an impact on brain development, organization and behaviour which cannot be

overcome with exogenous androgens due to complete androgen insensitivity, suggesting similar pathways would be impacted in human CAIS patients.

1.4.2 *Tfm* in Rats

Male rats with AIS (*Tfm* rats) retain a female phenotype and their abdominal testes produce normal to high levels of T with a normal T to DHT conversion rate; the first description of AIS in rats occurred in 1964 by Stanley and Gumbreck (Stanley, 1964; Bardin et al., 1970). Rat and mouse models of *Tfm* differ in terms of the type of mutation in the *Ar* gene. *Tfm* mice result from a spontaneous single base *deletion* which causes a frameshift mutation and loss of protein (Charest et al., 1991), whereas the mutation in rats involves a single base pair *substitution* in the *Ar* gene that does not change protein expression but severely affects binding of androgen ligand leading to a near-complete androgen-insensitive phenotype (Yarbrough et al., 1990).

Tfm rats also differ endocrinologically from WT male rat (Table 1.1). A study on the endocrine status of the *Tfm* rat compared against normal littermate WT rats was conducted at two different time points: 160 d and 350 d of age (Purvis et al., 1977). In both the younger and older group of *Tfm* rats, serum LH levels were significantly increased when compared to normal littermates, indicating a lack of negative feedback. The plasma levels of T were significantly increased in *Tfm* rats when compared to their littermates at both age groups as well (Purvis et al., 1977). Similarly, another study examined *Tfm* rats at ages 8-10 months and found that serum T and LH levels were significantly elevated in comparison to normal littermates. In this study the serum FSH level was significantly higher in *Tfm* rats than in normal littermates (Naess et al., 1976).

Tfm rats offer further evidence towards the *Ar* gene's contribution to the

masculinization of the nervous system and behaviour. Important for some aspects of sexual behaviour is the posterodorsal medial amygdala (MePD); this is smaller in *Tfm* rats – intermediate between that of WT males and females for both volume and soma size (Morris et al., 2005). Morris also looked at the suprachiasmatic nucleus (SCN), and found that in *Tfm* male rats, SCN volume and soma size were decreased compared to WT males. The locus coeruleus has important functions in anxiety (Redmond & Huang, 1979), and *Tfm* males have larger volumes and numbers of neurons in this area, similar to female WT rats (Garcia-Falgueras et al., 2005).

Behaviourally, *Tfm* rats differ from WT males (Table 1.2). In terms of spatial memory, males outperform females in both rodent and human models. *Tfm* rats displayed an intermediate pattern between WT males and females, taking longer to reach male-typical performances on the Morris Water Maze testing for spatial ability (Jones & Watson, 2005). As in *Tfm* mice, *Tfm* rats display reduced aggressive and sexual behaviour when compared to WT males (Beach & Buehler, 1977).

Table 1.1: Degree of function and endocrinology of Mouse *Tfm*, Mouse ARKO, and Rat *Tfm* compared to WT males.

	Degree of AR function	LH serum levels	FSH serum levels	T serum levels	References
Mouse *Tfm*	null	J: *Tfm* > WT A: *Tfm* > WT	A: *Tfm*> WT	J: *Tfm* = WT A: *Tfm* < WT	Lyon & Hawkes, 1970 Goldstein *et al.*, 1972 Murphy *et al.*, 1994 Jones *et al.*, 2003
Mouse ARKO	null	N/A	N/A	A: ARKO < WT	Notini, *et al.*, 2005
Rat *Tfm*	10-15% Ligand Binding Activity	J: *Tfm* > WT A: *Tfm* > WT	A: No Difference or *Tfm* > WT	J: *Tfm* > WT A: *Tfm* > WT	Purvis *et al.*, 1977 Naess *et al.*, 1976 Yarborough *et al.*, 1990

Table 1.1 shows the degree of function of the *AR* and LH, FSH, and T serum levels for mouse *Tfm*, mouse ARKO, and rat *Tfm*. Both the mouse *Tfm* and ARKO have null *AR* function, whereas the rat *Tfm* is not completely null. Adult (A) mice are approximately 6 wks of age and rats are older than 3 months. Juvenile mice (J) are between 3-6 weeks of age and rats are between 2 and 3 months. LH levels in the adult and juvenile *Tfm* mouse are significantly increased compared to WT males. FSH levels are also significantly elevated in adult *Tfm* mice, and T levels are significantly decreased in adult *Tfm* mice. Mouse ARKO males have significantly lower levels of T during adulthood. Rat *Tfm* males have significantly higher levels of LH and T during adulthood and as juveniles.

Table 1.2: Comparison of male-typical behaviours and anxiety-like behaviours across Mouse *Tfm*, Mouse ARKO, and Rat *Tfm*.

	Strain	Male-Typical Aggression	Male-Typical Sexual Behaviour	Anxiety	Spatial Memory	Social Interaction	References
Mouse *Tfm*	C57BL/6	*Tfm* < WT	*Tfm* < WT	*Tfm* >WT	N/A	N/A	Ono *et al.*, 1974 Rizk *et al.*, 2005 Zuloaga *et al.*, 2008
Mouse ARKO	(C57BL/6 x CD-1)	ARKO <WT	ARKO < WT	N/A	N/A	N/A	Sato *et al.*, 2004
Rat *Tfm*	King-Holtzmann	*Tfm* < WT	*Tfm* < WT	N/A	*Tfm* < WT	N/A	Meaney *et al.*, 1983 Beach *et al.*, 1977, Jones & Watson 2005

Table 1.2 shows behavioural results in male-typical aggression, sexual behaviour, anxiety, spatial memory and social interactions in mouse *Tfm*, mouse ARKO, and rat *Tfm*. All three groups had significantly lower levels of aggression and sexual behaviour compared to WT males. Mouse *Tfm* have significantly higher levels of anxiety compared to WT. *Tfm* rats display significantly lower levels of spatial memory on the Morris water maze when compared to WT rats.

1.4.3 A new mouse model for PAIS

Spontaneous and genetically-engineered rodent models of CAIS (*Tfm*) have been invaluable to deduce sex determination pathways and the broad physiological and behavioural roles of androgens, particularly in the context of male health. This research project describes a new mouse model of a PAIS-like phenotype that was first identified at the Jackson Laboratory following a mutagen-induced screen for novel heritable phenotypes. Male mice inherit this condition from their mother (Chr X-linked), they are infertile with intra-abdominal testes and are classified as pseudohermaphrodites; female carriers retain fertility and appear normal. The causative mutation is suspected to reside within the *Ar* locus on Chr X, although the exact molecular defect is still under investigation. The PAIS mutation was identified in C57BL/6J mice and the mutation is maintained on this strain background; this strain is not yet publically available through the JAX mouse catalogue, and this is the first published description of the PAIS mutant phenotype.

The goals of this research project address the following aims, in accordance with historical studies performed in *Tfm* rodent models: to create a rigorous description of the anatomy of PAIS male mice relative to normal fertile male mice of a C57BL/6J inbred strain background; to investigate perturbations of the HPG axis at the level of gonadotropin and serum T concentrations over two developmental time points, and to investigate the behaviour of adult male mice with the PAIS phenotype in terms of their male-typical, anxiety-like and social behaviours. This analysis will present new and relevant information to the field of gender disorders that is particularly relevant to patients with the PAIS condition.

1.5. Rationale and Hypotheses:

Interference with androgenic signaling via mutations in the androgen receptor (*Ar*) gene has severe anatomical, endocrinological, physiological and behavioural consequences in CAIS rodent models. The PAIS mouse model under study has anatomical features that are intermediate between male and female characteristics, suggesting a significant disruption in androgenic signaling results from this unique Chr-X linked mutation. Three experimental hypotheses will be examined in this mouse model of PAIS:

Hypothesis 1: A major disruption in androgenic signaling will interfere with normal negative feedback regulation of the hypothalamic-pituitary-gonadal axis. This will be reflected by a change in serum steroid (T) and gonadotropins (LH and FSH) concentrations relative to male mice that do not carry this mutation.

Hypothesis 2: A major disruption in androgenic signaling will influence typical male behaviours. This will manifest as reduced aggression and sexual interest.

Hypothesis 3: A major disruption in androgenic signaling will increase anxiety-like behaviours.

Chapter 2 – MATERIALS AND METHODS

2.1 Mice

2.1.1 Strain descriptions and husbandry

A colony of C57BL/6J mice carrying the PAIS mutation was identified at The Jackson Laboratory (Bar Harbor ME) following a mutagenesis screen for new mutations. Mice were transferred to Memorial University in 2007 and the breeding colony was housed in the Specific Pathogen Free Barrier of the Health Sciences Centre. In the absence of a specific genotyping assay, female mice were progeny tested with C57BL/6J males to determine their PAIS mutation carrier status based on the identification of PAIS mutant males in their offspring. For female carriers, the mutation transmission ratio to male offspring was close to the expected rate of 50% (48%), indicating that embryonic lethality in male mutation carriers was not significant.

All endocrinological and behavioural studies were performed on WT or PAIS mutant males maintained on the C57BL/6J inbred background, with a preference for litter-mate pairs for behavioural analyses. In subsequent text, the nomenclature assignment shall be C57BL/6J^{WT} and C57BL/6J^{PAIS}. In one case, C57BL/6J female carriers of the PAIS mutation were mated to males of the SJL/Bm inbred strain, to create hybrid (C57xSJL)F1 male offspring for further male-typical behavioural studies. In subsequent text, the nomenclature assignment shall be (C57xSJL)F1^{WT} and (C57xSJL)F1^{PAIS}. A/J and FVB/NJ inbred mice were purchased from the Jackson Laboratory; A/J males were used for anxiety and resident-intruder behavioural analyses, and albino females (A/J or FVB/NJ) were used in the tests of sexual interest for

C57BL/6J or (C57xSJL)F1 males, respectively. Female mice were not littermate pairs and had a C57BL/6J background; they were used as a comparison group for phenotype in the analysis of anal-genital distances and body weight. They were also incorporated into the genotyping assay.

2.1.2 Animal maintenance and IACC approvals

All mice had access to LabDiet food (22% protein, 5% fat, 5% fiber, 6% ash, with wheat filler as remainder) and fresh tap water *ab libitum*. Offspring were housed in same sex littermate groups, weaned between 19-21 d of age; rodent cages were 11"L x 7"W x 5"H with corn-cob bedding material (Bed-O-Cobs (LabDiet, IN, USA.). PAIS mutants were housed with WT males siblings under a 12 h:12 h light/dark cycle with the light cycle commencing at 8 am and terminating at 8 pm. All protocols were approved by the Institutional Animal Care and Use Committee of Memorial University.

2.1.3 Phenotype Assignment

Mice were weaned between 19-21 d of age, with sex determined by anal-genital distance (longer in males than females), and/or presence of mammary chain development in females. Sexing the PAIS mutant weanlings was often challenging at typical weaning age, due to their intermediate anal-genital distance and the presence of fur pigmentation that resembles the mammary chain of females. At such an early age PAIS mutants were not easily recognizable, and thus may have been originally incorrectly assessed as females; PAIS mutants were clearly identifiable after a week or so post-wean and we were then able to separate them into the correct male cages. All WT and PAIS mutants were of a C57BL/6J background (black fur) and they were indistinguishable from one another unless their ventral side was examined. As the animals age to 5 or 6 wks, sex

determination is much simpler and sometimes weaned litters were re-visited to rectify sexing errors. All animals were inspected at necropsy for the definitive presence of testes and the absence of male internal reproductive structures (epididymis, seminal vesicles) to confirm PAIS carrier status.

2.2 Serum Collection and Body Characteristics

Serum was collected between 9 am -11 am, from C57BL/6J^{PAIS} mutants and C57BL/6J^{WT} controls at 30 d and 50 d of age, when mouse serum gonadotropin levels and gonadal steroid levels peak, respectively (Selmanoff et al., 1977). Trunk blood (~1 ml) was collected into sterile 1.5 ml microcentrifuge tubes following decapitation. The blood samples sat on ice for ~2 h followed by a spin in the centrifuge for 10 min (13,000 rpm). Serum (~100-150 μl) was then collected from the top supernatant layer into clean and labeled tubes and stored at -20 °C until assay. Assays were performed at the University of Virginia Ligand Core Laboratory for the following serum components: T, LH, and FSH. Samples were frozen (-20 °C) and then packed on dry ice in Styrofoam containers and numbered so that the University of Virginia Ligand Core was blind to the phenotypic groupings. They were couriered to Virginia with pre-determined dilution instructions. Extra samples for each assay were sent in order to test the out the dilutions. Once dilutions were verified in their reportable range, test samples were assayed; the average reportable range for the mouse FSH assay is 2-25 ng/ml, LH is 0.07-37.4 ng/ml, and T is 10-1000 ng/dl. Mouse LH was measured using an immunoradiometric assay, whereas mouse T and FSH were measured using a radio-immunoassay. The data were verified using a calibration curve for each assay.

Following serum collection, the following measurements were taken: anal-genital distance, body weight, testes pair weight, preputial gland pair weight, and kidney pair weight. Body weights were measured using a precision balance whereas an analytical balance was used to measured the weights of organ pairs. Anal-genital distance was measured using a plastic millimeter ruler.

2.3 Polymerase Chain Reaction (PCR) Assay for Sex Chromosome Determination

2.3.1 DNA extraction from tail tip

Crude tail DNA was used for the PCR analyses. Tail tips (~5 mm) were obtained from male, female, and mutant mice and were individually placed into 1.5 ml microcentrifuge tubes and 500 µl of 50 mM NaOH was added to each tube. The tubes were placed on a heat block set at 95 °C for 10 min. Following removal from heat, 50 µl of 1M Tris-HCl (pH 8.0) solution was added to each of the tubes. Samples were inverted, mixed using a vortex mixer and then centrifuged at 13,000 rpm in the microcentrifuge. Approximately 500 µl of liquid was removed and placed into clean, labeled 1.5 mL tubes and the DNA samples were stored in the -20 °C freezer until assay.

2.3.2 Polymerase Chain Reaction Conditions

PCR was conducted on C57BL/6J^{PAIS} genomic DNA to assess sex chromosome genotype, using C57BL/6J^{WT} male and C57BL/6J female samples as positive controls. Amplification was performed using a cocktail of 10 µl mastermix (MasterTaq Kit '5 Prime' Inc, Gathersburg MD) created in a clean labeled 1.5 mL tube, using the following reagents: 7.3 µl H_2O, 1 µl TaqMaster PCR enhancer (heated to 65 °C), 0.2 µl of 10 mM dNTP mix (Qiagen, Valencia CA), 1 µl MasterTaq buffer with Mg^{2+}, 0.22 µl of forward

and reverse primers, and 0.05 µl Taq DNA polymerase. 1 µl of tail-extracted DNA was added to an aliquot of mastermix for each sample and primer pair assessed. Thermal cycling was done in an Applied Biosystems Verti^{TM} Thermal Cycler (Applied Biosystems Inc.) with the following profile: 97 °C for 30 sec; 39 cycles of 94 °C for 15 sec, 55 °C for 30 sec, and 72 °C for 30 sec; 72 °C for 10 min (Table 2.1). To confirm karyotic sex, two primers, [SMC-1,TGAAGCTTTTGGCTTTGAC] and [SMC-2, CCGCTGCCAAATTCTTTGG] were used to distinguish the Chr X and Chr Y homologues of the *SmcX* and *SmcY* genes, respectively (Mroz et al., 1999). These primers were ordered from IDT Inc. (Coralville, IO). Following PCR amplification, 1 µl of loading dye was added into each of the PCR tubes and run against 8 µl of 100 bp ladder.

2.3.3 Gel Electrophoresis

Horizontal gel electrophoresis was used to determine the relative size of the amplified products. PCR products were run on a 2% agarose gel comprised in 1 x TRIS-Borate-EDTA (TBE) buffer. The solution was heated in a microwave and 2 µl of 10 mg/ml ethidium bromide was added to detect DNA; the solution was poured into the gel platewith horizontal comb in place (agarose, ethidium bromide, and TBE, Sigma Aldrich Co. (St. Louis, MO)). The gel was electrophoresed at 100 V for approximately 1 h after which fluorescent bands were visualized using the Syngene UGENIUS gel documentation system (Discovery Scientific Inc, Vancouver BC).

2.4 Androgen Sensitivity Test

Generally, the androgen sensitivity test measures the organ responses to

exogenous androgen following a period of androgen deprivation, achieved by the testes removal (castration). The specific protocol was as follows: Male pairs were anesthetized with isoflurane gas and surgically castrated, which required the removal of intra-abdominal testes from C57BL/6J^{PAIS} mutant males. Post-surgical analgesia was provided using 5 mg/kg of carprofen administered i.p after each surgery. Castration was followed by a 2-week recovery after which a 10 mm silastic tubing capsule (Fisher Scientific, Ottawa ON) containing solid packed T capped by glass beads or an empty silastic capsule was subcutaneously implanted in the back during a second surgery (Fig 2.1). Testosterone (4-androsten-17β-ol-3-one) was purchased from Steraloids, (Newport, RI) and licensed for use through the Office of Controlled Substances, Health Canada. Four groups of animals were assigned as follows: T-implanted PAIS males (n = 9) T-implanted WT males (n= 9), Empty-capsule PAIS males (n = 8) and Empty-capsule WT males (n = 8). Two wks following capsule implantation, the mice were euthanized and the body weight and androgen responsive organs (kidneys, preputial glands, seminal vesicles) weights recorded. Statistical analyses were performed using a student's t-test for independent samples for the seminal vesicles, and a two-way ANOVA for both the preputial glands and kidneys, with significance set at $p<0.05$.

2.5 Behavioural Tests

All of the behavioural testing was videotaped. Behaviours were manually scored from observation of the video. The Ethovision XT system for use of computerized behavioural tracking was used for two of the behavioural tests: Elevated Plus Maze (EPM) and the Open Field test (OF).

2.5.1 Resident Intruder Test

Twenty-five litter-matched male pairs (C57BL/6J^{WT} and C57BL/6^{PAIS}) between 10-12 wks of age were examined for aggressiveness. Prior to testing, males were singly housed for one week without a bedding change. On the day of the test, males were brought to the testing room 30 min prior to testing. The test room was dimly lit and tests were performed during the late afternoon (mouse holding room light cycle) in the test mouse's homecage. An unfamiliar intruder male mouse (albino A/J) was introduced into the homecage and their behaviour was observed for 10 min over three consecutive days at the same time of day; after 10 mins the intruder mouse was removed on each of the three days. The procedure was the same each day (Raskin et al., 2009) (Fig 2.2). When mice are subjected to multiple aggression encounters over a period of days, the aggression builds (Raskin et al., 2009); because we were looking to measure a decline in aggressive behaviour we recorded only the third day of the aggression trials in order to have the most sensitive assay. For each trial, the test males interacted with a different intruder so no familiarization would occur across trials. The total amount of time spent fighting and the number of individual fights that occurred were recorded. Fighting is described as wrestling behaviour and harmful contact behaviour towards another mouse defined by the single observer. Following each trial, the intruder males were removed from the test male's homecage, leaving them once again in isolation. The observer was blinded to the phenotype of the test mouse, and manual scoring of the resident intruder test was done by the same observer. Statistical analyses were performed using a student's t-test for paired samples, with a significance level set at $p < 0.05$.

On a separate occasion, the resident-intruder paradigm was repeated with WT

and PAIS mutation carriers on a different mouse strain background. In this case, 10 pairs of (C57xSJL)F1^{WT} and (C57xSJL)F1^{PAIS} males were examined, using A/J strain males as the intruders. In this test, nine pairs of males were litter-mates, and only one pair was obtained from two different litters, and thus experienced a different maternal environment. The 10 pairs were analyzed using a student's t-test for unpaired samples, with a significance level set at $p < 0.05$.

2.5.2 Sexual Interest Test

Twenty-five litter-matched pairs of C57BL/6J^{WT} and C57BL/6J^{PAIS} males between 10-12 wk of age were examined for sexual interest. This test followed the resident-intruder test with a gap of either 1 or 2 d while the males remained singly housed. The males were brought to the test room 30 min prior to testing. The sexual interest test was performed in the test mouse's homecage, which had the bedding unchanged for over 7 d. An unfamiliar female in estrus was introduced into the homecage and their behaviour was recorded over one 30 min trial. To ensure sexual receptivity, the A/J female mice were induced into estrus by receiving a subcutaneous injection of estradiol dissolved in peanut oil (25 μg/0.1ml) at 48 h and 24 h prior to testing (Edwards, 1968; Soukhova-O'Hare et al., 2007). A third subcutaneous injection of progesterone dissolved in peanut oil (1 mg/0.1ml) was given 4-5 h before the test (Fig 2.3). The number of successful mounts and the time spent mounting were recorded. The observer was blinded to the phenotype of the test males, and manual scoring by a single observer took place. Statistical analyses were performed using a student's t-test for paired samples, with a significance level set at $p < 0.05$.

On a separate occasion, the sexual interest paradigm was repeated with WT and PAIS mutation carriers on a different mouse strain background. In this case, 10 pairs of (C57xSJL)F1^{WT} and (C57xSJL)F1^{PAIS} males were examined, using available albino FVB/NJ females as the partners, following the same estrus induction scheme described earlier. In this test, nine pairs of males were litter-mates, and only one pair was obtained from two different litters. The 10 pairs were analyzed using a student's t-test for unpaired samples, with a significance level set at $p < 0.05$.

2.5.3 Tests of Anxiety

A unique cohort of twenty-one litter-matched pairs (C57BL/6J^{WT} & C57BL/6J^{PAIS}) of males between 10-12 wk of age was examined using three well known tests for anxiety-like behaviours: elevated plus maze (EPM), open field (OF), and the light/dark box (LDB). Male mice were housed as litter-mate pairs in the Biotechnology Buidling (Memorial University of Newfoundland) for one week prior to testing after transfer from central animal care facilities. Twenty A/J male mice (10-13 wk) were also tested in each of the three anxiety paradigms as a positive control strain; A/J mice have been reported to exhibit more anxiety-like behaviours than C57BL/6J^{WT} mice (Whalsten & Crabbe, 2003). Tests were performed on three consecutive days in the following order: EPM, OF, and L/D, and the testing order of individual mice was the same everyday. A/J albino male mice were colored slightly with black marker to contrast with the white EPM and OF anxiety apparati, facilitating computer tracking. For consistency all mice being tested were stroked on the back with marker or a marker with no ink. The observer was blind to the genotype of the test mouse when it came to C57BL/6J^{WT} versus C57BL/6J^{PAIS}; it was obvious when the A/J mice were being scored because their coat

color was white. Scoring was performed both manually and using Ethovision XT computerized tracking. When manual scoring of the three tests of anxiety was performed a strict guideline was enforced: all four paws of the mouse (not including the tail) had to completely be in either the open arm, centre zone, or light area in order to be counted. When Ethovision XT was used in order to evaluate behaviour, the mouse body was divided into three points (head, middle and base) and when either of these points were in the designated areas, it was counted as an entry. The consequence of this is an increase in number counts for the computerized tracking relative to manual scoring.

2.5.3.1 Elevated Plus Maze

Approximately 30 min prior to testing, males were brought to the testing room. Tests were performed in an EPM apparatus consisting of two closed and two open arms (30.5 cm long x 5.2 cm wide). The walls of the closed arms were 14.3 cm high and the lip around the perimeter of the open arms was 0.6 cm high. The center square was 5.2 x 5.2 cm and the entire maze was elevated 47 cm from the ground (Fig 2.4). Each subject spent one 5 min trial in the EPM. There were two identical mazes, therefore two animals could be tested at one time. Tests were run in dim red illumination and tails were marked with a permanent marker for identification. Mice were placed in the center dead zone to start the test and after each trial their fecal boli were counted, as an extra measure of anxiety. Between trials, the EPM was cleaned with a solution of 5% ethanol and dried before the next test subject. Time spent in the open and closed arms of the maze and the number of entries into the arms were recorded. Manual scoring was performed for analyses of time and frequency in the open arms, however scoring was further validated when a behavioural tracking system (Ethovision XT) became available for computer tracking.

The mean velocity of the animal and the total distance traveled was also recorded with the automated tracking system. Statistical analyses were performed using a student's t-test for paired samples, with a significance level set at $p < 0.05$, and a one-way ANOVA when the analyses included A/J mice as the third group.

2.5.3.2 Open Field

The open field (OF) apparatus is a white box, larger than the homecage, with a 37.2 cm x 37.2 cm floor and 20 cm high walls (Fig 2.5). Each test subject spent one 10 min trial in one of the two identical open field boxes. Two subjects were tested at one time. Tests were run under dim red illumination and all tails were marked with permanent marker for identification. Mice were placed in the centre of the apparatus to start the trial, and their fecal boli were counted when the trial was finished. In between trials, the apparatus was cleaned with a 5% solution of ethanol. PAIS and WT males were randomly assigned between the two identical OF apparati. Recorded variables were time spent in the centre zone and frequency entering the centre zone (which comprised 50% of the floor) and the periphery surrounding the centre (comprised of a 3 cm square from the wall of the box). Measures of mean velocity as well as total distance travelled were also recorded for each animal. Between the two zones was a 'dead' zone in which the animal was not tracked. Data were collected first with manual scoring and then again using the computerized system Ethovision XT for further analyses. Observers were blinded to the genetic status of the mice; this is only true for C57BL/6J^{WT} versus C57BL/6J^{PAIS} and not for A/J mice because their coat color is white. Statistical analyses were performed using a student's t-test for paired samples, with a significance level set at $p < 0.05$, and a one-way

ANOVA was used when A/J mice were used in the analyses as a third group.

2.5.3.3 Light/Dark Box

The LDB was run on the third consecutive day of behavioural testing. Animals were housed as litter-mate pairs and brought to the test room 30 min prior to testing for acclimatization. The LDB consisted of two equal size covered grey boxes, 19.9 cm x 19.9 cm x 15 cm, connected by a small enclosed tunnel 6.6 cm x 10.1 cm x 7.2 cm. One of the boxes is completely opaque (dark box), and the other box had a transparent top with breathing holes (light box) (Fig 2.6). A 40 watt bulb was positioned over the light box. Each test subject was placed in the centre of the light side to begin the 5 min trial. There were four LDB setups available so 4 animals were tested at one time. Animals were tail marked with a black permanent marker to keep track of individuals. The trial was run under dim room lighting, and the apparatus was cleaned between runs using a 5% ethanol solution and dried. Fecal boli were counted following each run. The number of transitions into the light area and the amount of time spent in the light area were recorded. Scoring of these measures was manual by one observer blinded to the genetic status. Statistical analyses were performed using a student's t-test for paired samples, with a significance level set at $p < 0.05$, and a one-way ANOVA was used when A/J mice were included in the analyses as the third group.

2.5.4 Test of Social Interaction

The same twenty-one pairs C57BL/6J^{WT} and C57BL/6J^{PAIS} males were also tested on social interaction. Following the three consecutive days of anxiety testing, the Social Interaction Open Field (SI-OF) test was conducted. Mice were tested in the same testing order as the previous tests and were still pair housed.

The SI-OF apparatus is a large box with a 47 cm x 47 cm floor, and 48 cm high walls. A wire mesh box holding the stimulus animal within the large open box is 13.5 cm x 9.0 cm and is 10 cm high; the walls are made of Plexiglas (5 mm thick) and rise up 16 cm in height above the mesh (Fig 2.7). The test was run under dim room lighting conditions, and two identical apparati were used so two animals were tested at the same time. Each test subject underwent one 5 min trial. For the initial 2.5 min the test subject was alone (Trial A). The animal was then removed while a stimulus animal was placed in the wire mesh box within the interaction zone. Once the stimulus animal was in place, the test mouse was put back into the box for the final 2.5 min (Trial B). The amount of time and number of entries into the interaction zone and the non-interaction zone for both Trial A and Trial B, total distance travelled, mean velocity, and the number of head points towards the stimulus animal's cage while in the interaction zone for both trials. After Trial B, the box was cleaned with a solution of 5% ethanol and dried. The tracking system Ethovision XT was used for scoring of social behaviours. Statistical analyses were performed using a two-way ANOVA (genotype x trial), with a significance level set at $p < 0.05$.

2.6 Statistical Analysis

Data were expressed as mean ± standard error of the mean as absolute values and was analyzed using the statistical program Prism (GraphPad Software Inc, version 5.0b, La Jolla, CA). A student's paired t-test ($p<0.05$) was used to determine significant differences of body weights, organs weights and anal-genital distances. The results of the serum assays was analyzed using a student's t-test for independent samples. Statistical

analyses of behavioural tests were analyzed using a student's paired t-test ($p<0.05$). When all three groups ($C57BL/6J^{WT}$, $C57BL/6J^{PAIS}$, and A/J) were compared, a one-way ANOVA statistical test was used, followed by Tukey's multiple comparison test. Both the androgen sensitivity test and the social interactions behavioural tests were analyzed using a two-way ANOVA ($p<0.05$).

Table 2.1: Polymerase Chain Reaction (PCR) Conditions

Reaction Volume	Initial Step	39 Cycles of:			Final Step
10.77 µL	HOLD	Denature	Anneal	Extend	HOLD
	30 sec	Target: 94°C	Target: 55°C	Target: 72°C	10 min
	97°C	Hold: 15 sec	Hold: 30 sec	Hold: 30 sec	72°C

Table 2.1 shows the conditions of the polymerase chain reaction assay for sex chromosome determination. The reaction volume, initial step, cycles, and final step are described.

Figure 2.1: Paradigm for Androgen Sensitivity Test

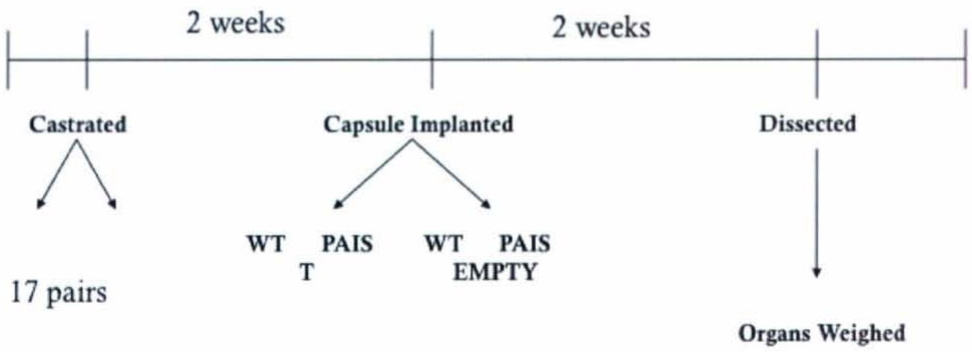


Figure 2.1 shows the paradigm for the androgen sensitivity test. Mice were orchidectomized and given 2 wk to recover from the surgery, at which point an empty capsule or a capsule containing T was implanted into WT males and PAIS mutants. After another period of 2 wk, the mice were euthanized and organs weighed.

Figure 2.2: Resident-Intruder Paradigm

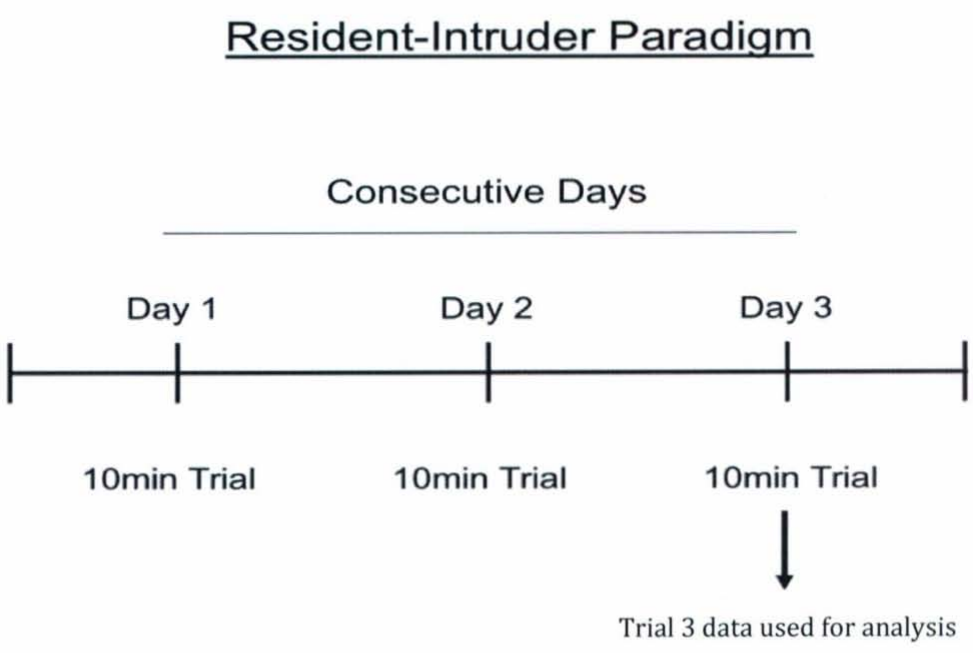


Figure 2.2 shows the paradigm for the resident-intruder behavioural test. Trials occurred on three consecutive days with mice undergoing a 10 min trial each day using a novel intruder male stimulus mouse in their homecage. Aggression builds over trials, so only the data from day 3 was analyzed in order to obtain the most sensitive assay.

Figure 2.3: Sexual Interest Paradigm

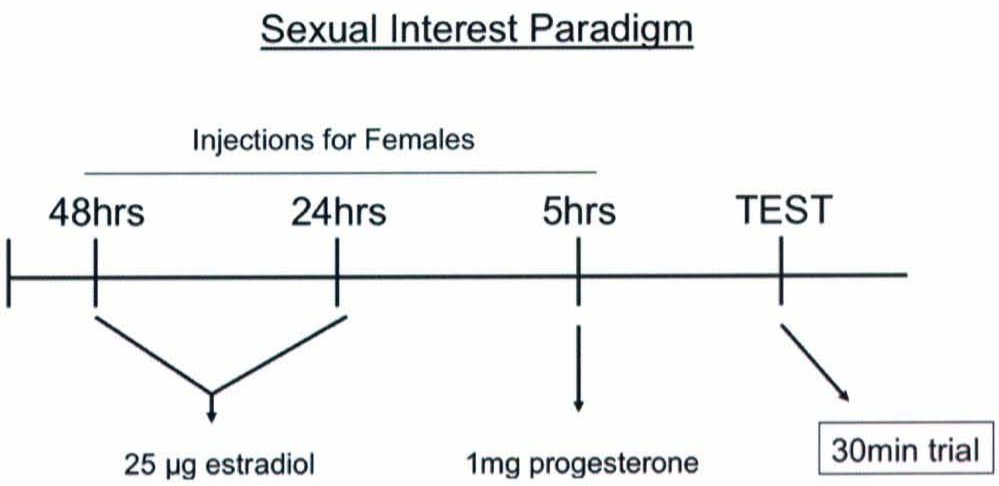


Figure 2.3 shows the sexual interest behavioural paradigm. At 48 and 24 h prior to testing the females were injected with 25 µg of estradiol and at 4-5 h prior, 1mg of progesterone. The test was run as a single 30 min trial in the male mouse's homecage.

Figure 2.4: Diagram of Elevated Plus Maze

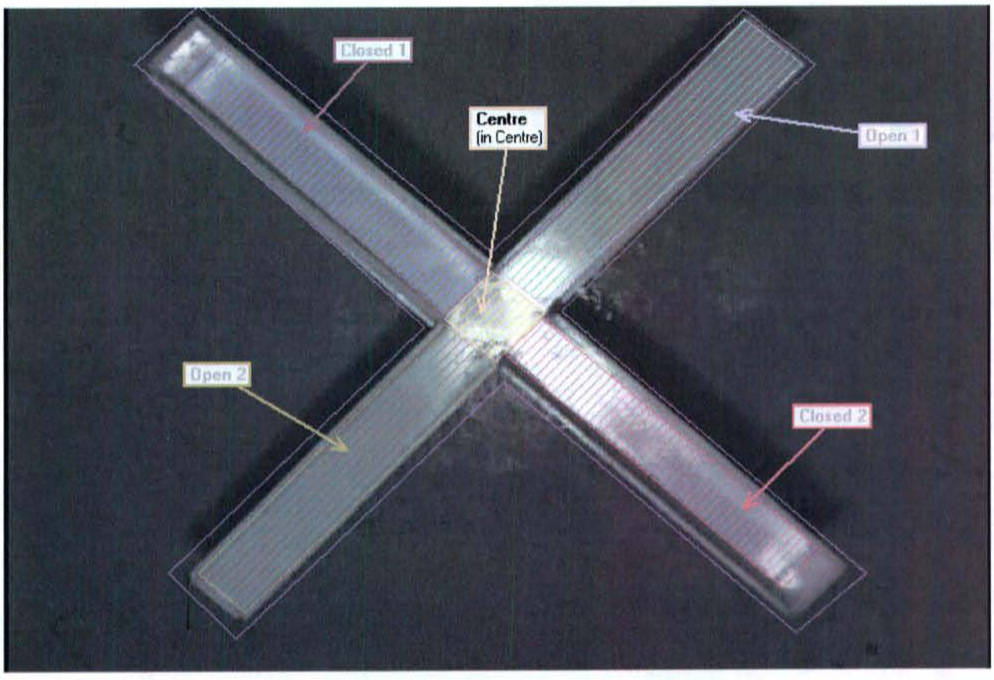


Figure 2.4 shows the elevated plus maze apparatus. The two arms on the bottom right and top left are closed, and the top right and bottom left are open. The center dead zone is a square in the middle of the maze.

Figure 2.5: Diagram of Open Field

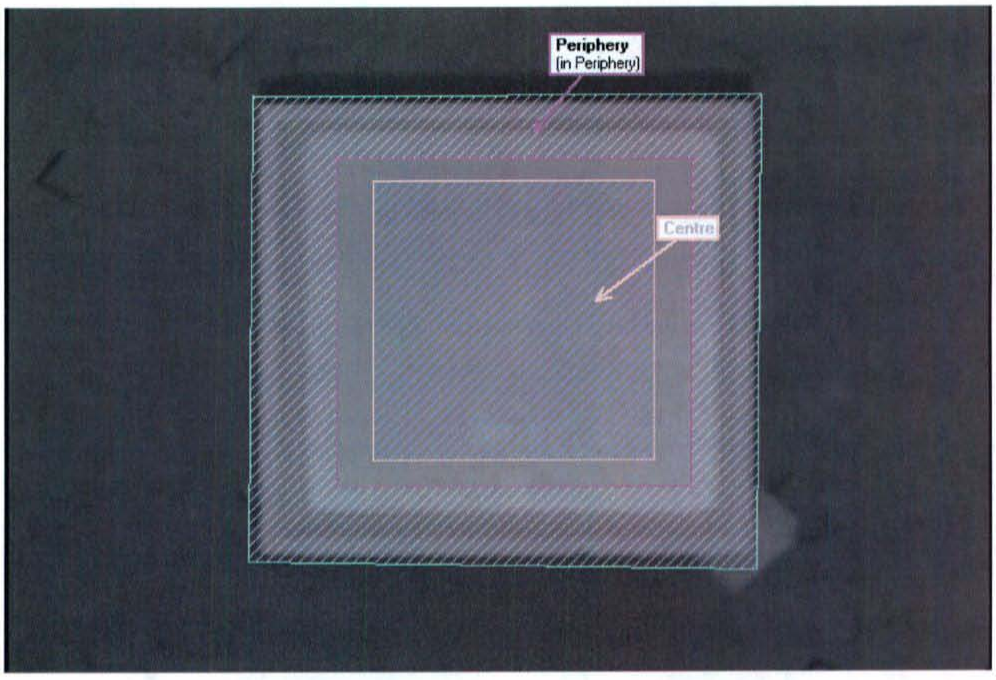


Figure 2.5 shows the open field apparatus. The centre square exists in the middle of the open field and the periphery zone lines the edges of the walls. In between the two zones is a dead zone.

Figure 2.6: Diagram of Light/Dark Box

Figure 2.6 shows two light/dark box apparati side by side. Each apparatus contains an open air lighted compartment connected to an enclosed dark compartment via a small tunnel.

Figure 2.7: Diagram of the Social-Interaction Open Field

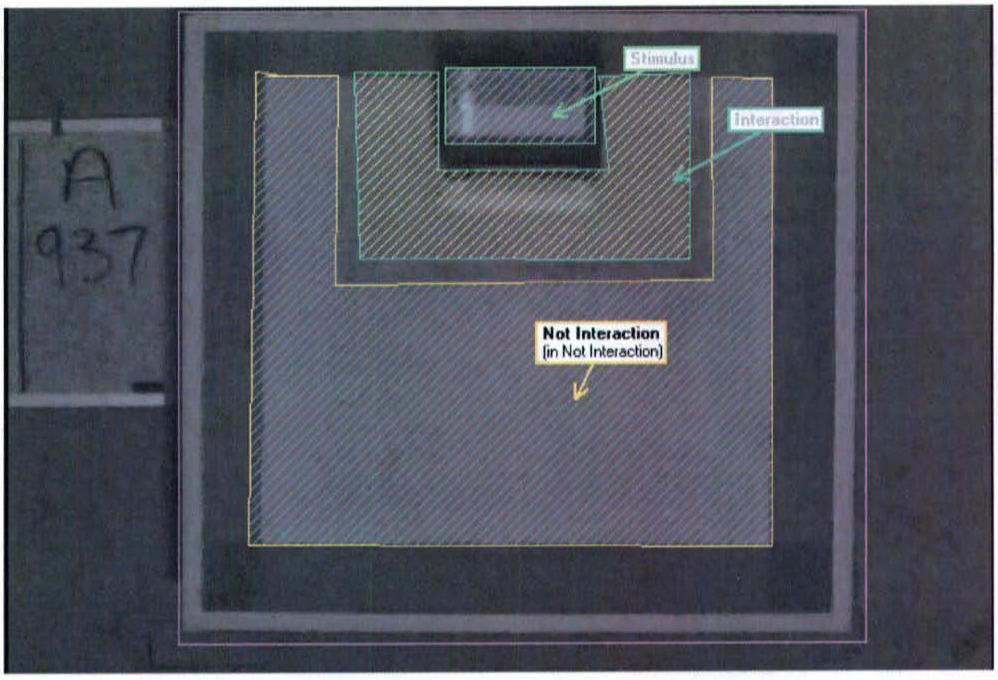


Figure 2.7 shows the social interaction open field apparatus. The non-interaction zone, interaction zone, and the small mesh cage where the stimulus animals were kept during Trial B are shown.

Chapter 3 – RESULTS

3.1 Anatomy

Key differences in the external anatomy of the male C57BL/6J^{PAIS} and C57BL/6J^{WT} mice were useful for phenotype discrimination. On the black coat of the C57 inbred background, C57BL/6J^{PAIS} males have mammary chain pigmentation on their ventral side and they lack a scrotum (Fig 3.1). This is consistent representative external anatomy as upwards of 250 animals have been observed. Furthermore, the anal-genital distance of male and female C57BL/6J^{WT} is visibly different from C57BL/6J^{PAIS} male mice. When measured at 50 d of age, C57BL/6J^{PAIS} males had an intermediate phenotype between C57BL/6J^{WT} males and females with a significantly larger distance than females and a significantly smaller distance than normal males (Table 3.1). WT versus PAIS male phenotype assignments were always confirmed at necropsy by an internal examination.

Body weights (g) of all three groups were examined and PAIS mutants were found to be significantly heavier than female WT mice at 50 d of age (Table 3.1). There was no significant difference in the body weights of PAIS mutants and WT males at 50 d. Despite the presence of intra-abdominal testes, C57BL/6J^{PAIS} males are infertile due to their missing internal male reproductive structures, including the seminal vesicles, prostate lobes, vas deferens, and the epididymis (Fig 3.2). Between 10-12 wks of age, the androgen-responsive organs present in both C57BL/6J^{WT} and C57BL/6J^{PAIS} males were weighed for comparison. The testes, kidneys, and preputial gland pair weights normalized to body weights were significantly smaller inC57BL/6J^{PAIS} compared to C57BL/6J^{WT} mice (Table 3.2). There was no significant difference in body weight

between C57BL/6J^{WT} and C57BL/6J^{PAIS} mice at 10-12 wks of age (Table 3.2), consistent with measurements at 50 d (~7 wks).

3.2 Serum Assay

Data was collected on the serum levels of T, LH, and FSH for both C57BL/6J^{WT} and C57BL/6J^{PAIS} males (Table 3.3). When each phenotype was analyzed for T there were no significant differences between groups at either 30 d or 50 d time points. At 30 d there were no significant differences in LH between phenotypes, however at 50 d C57BL/6J^{PAIS} males had significantly higher levels of LH compared to C57BL/6J^{WT}. C57BL/6J^{PAIS} males had significantly higher levels of FSH at both 30 d and 50 d compared to C57BL/6J^{WT} males (Table 3.3).

3.3 Sex Chromosome Genotyping Assay

Using a PCR based assay for two genes specific for Chr X and Chr Y, *Smcx* and *Smcy*, it was determined that the C57BL/6J^{PAIS} males were karyotypically identical to WT males, possessing both Chr X and Chr Y. Fig 3.3 depicts the electrophoretic pattern of two independent female DNA samples showing a single band to represent the *Smcx* gene, whereas two independent DNA samples from C57BL/6J^{WT} and C57BL/6J^{PAIS} males share the same doublet pattern for *Smcx* and *Smcy* amplification products.

3.4 Androgen Sensitivity Test

The testing paradigm described was performed on adult male mice, thus it would not be expected that exogenous T could rescue the anatomical reproductive defects of the C57BL/6J^{PAIS} males. Body and organ weights were measured for four groups of males characterized by phenotype (WT vs. PAIS) or treatment (T supplementation vs. empty capsule) (Table 3.4). Organ weights were normalized to body weights due to the

high variability in the age range (11-23 wks) of the littermate pairs at the time of surgery.

Although C57BL/6J^{PAIS} males do not develop seminal vesicles, this organ in WT males was used as a positive control for the experiment. As shown in Table 3.4, C57BL/6J^{WT} males who received T capsules had significantly larger seminal vesicles, and therefore larger ratios of seminal vesicle weight per body weight than WT males who received empty capsules, and so the described paradigm was useful for measuring androgen sensitivity. Subsequently, the kidneys and preputial gland pair weights were compared between the C57BL/6J^{PAIS} and C57BL/6J^{WT} T and empty capsule groups in order to determine if T had an equal effect on organ re-growth. Despite the equal dose and length of exposure to T, the C57BL/6J^{PAIS} group still had significantly smaller preputial glands normalized to body weight than C57BL/6J^{WT} males (Table 3.4), but no significant difference was seen in kidney weights. Even though the PAIS T treatment group did not reach the organ size of the WT T group, there was a significant growth response to T compared to the empty capsules. Therefore it can be concluded from this assay that C57BL/6J^{PAIS} males have a partially androgen-insensitive phenotype. The seminal vesicles were analyzed using a student's t-test for independent samples ($p<0.05$), and the kidneys and preputial glands were analyzed using a two-way ANOVA, $p<0.05$.

3.5 Behavioural Tests

3.5.1 Resident Intruder Test

C57BL/6J^{PAIS} and C57BL/6J^{WT} males did not differ significantly in the amount of time spent fighting or in the number of fights (Fig 3.4 A, B). The behaviour observed during the resident intruder test was minimal, and observing significant differences between groups was difficult due to the lack of aggression in either group. This test was

followed up using a more aggressive SJL inbred strain cross with the C57BL/6J female carriers. When the PAIS mutation was crossed onto the (C57 x SJL)F1 hybrid background, the number of aggressive acts generally increased in both WT and PAIS male mutation carriers. Furthermore, using the same resident-intruder test paradigm, the (C57 x SJL)F1^{WT} were significantly more aggressive compared to (C57 x SJL)F1^{PAIS} males. They spent significantly more time fighting (Fig 3.5 A) and had significantly more fighting bouts (Fig 3.5 B), indicating the PAIS mutation has a significant impact to reduce aggressive behaviour. Therefore the strain background switch improved the sensitivity of the assay in order to measure a decline in aggressive behaviour.

3.5.2 Sexual Interest Test

Comparison of the time spent mounting a novel female in estrus and the number of mounts showed significant differences between C57BL/6J^{PAIS} and C57BL/6J^{WT} males (Fig 3.6 A, B). The behaviour observed during the sexual interest test was minimal and observing any differences between groups was difficult due to the lack of activity in either group; therefore the (C57 x SJL)F1 males were examined for sexual interest. Using the same sexual interest paradigm, (C57 x SJL)F1^{WT} were significantly more active in sexual behaviours when compared to the (C57 x SJL)F1^{PAIS} males. WT males spent significantly more time mounting (Fig 3.7 A) and attempted more mounts (Fig 3.7 B), indicating the PAIS mutation has a significant impact to reduce sexual behaviour.

3.5.3 Anxiety Tests

When Ethovision was used to evaluate tests of anxiety, measures of time and frequency are significantly higher than when manual scoring was used for the exact same set of behaviours; this is due to a difference in the scoring paradigm. When I scored the

behaviours manually I set a paradigm for myself that only when the 3 points of the mouse (base, middle, head) had completely crossed into the area I would start timing or count it. However when I set up the Ethovision program's paradigm I allowed for all 3 points to be counted separately, meaning that anytime either the base, middle, or head crossed into the area it would be counted. This explains why the Ethovision count for the same videos is significantly higher. Regardless of using the 3 points as a whole or separate, the data were consistent across manual and computerized scoring. We included a cohort of A/J male mice (n=19) in all three tests of anxiety as a positive control strain.

3.5.3.1 Elevated Plus Maze

(A) Manual Scoring

A/J male mice spent significantly less time in the open arms compared to C57BL/6J^{WT} (Fig 3.8 A). C57BL/6J^{PAIS} mice also spent significantly less time in the open arms compared to C57BL/6J^{WT} mice (Fig. 3.8 A). Entries into open arms were also measured. A/J mice entered into open arms significantly fewer times (Fig 3.8 B). The number of entries to open arms did not differ between C57BL/6J^{WT} and C57BL/6J^{PAIS} males (Fig. 3.8 B).

(B) Computerized Scoring

Similar results were calculated using Ethovision XT in the EPM test: C57BL/6J^{PAIS} males spent less time in the open arms (Fig 3.9 A) and entered the open arms significantly fewer times (Fig 3.9 B) when analyzed by a student's t-test. A/J males were not included in the computerized analysis due to their white coat color which was not well discriminated by the tracking software versus the white backdrop of the EPM apparatus. Although black markings were applied to the back of A/J males, the

computerized system failed to track the A/J males accurately.

No significant differences were found between C57BL/6J^{WT} and C57BL/6J^{PAIS} males for the time spent in the closed arms (Fig 3.10 A) or number of entries into closed arms (Fig 3.10 B). Total distance (cm) and mean velocity (cm/s) were compared, and no significant differences were found in either measure (Fig 3.11 A & B).

3.5.3.2 Open Field

(A) Manual Scoring

A/J male mice spent significantly less time in the centre zone compared to C57BL/6J^{WT} (Fig 3.12 A). C57BL/6J^{PAIS} mice did not differ from C57BL/6J^{WT} for amount of time spent in the centre zone (Fig 3.12 A). A/J mice entered into the centre zone significantly fewer times compared to C57BL/6J^{WT} males (Fig 3.12 B). No significant difference on number of entries to the centre zone was found between C57BL/6J^{WT} and C57BL/6J^{PAIS} mice (Fig 3.12 B).

(B) Computerized Scoring

When the centre zone was reduced from 64% to 50% of the entire box for more strict analyses using computerized tracking, identical results were seen for both time and number of entries into the centre zone between C57BL/6J^{PAIS} and C57BL/6J^{WT} males. No significant differences between groups was seen for amount of time spent in the centre zone (Fig 3.13 A) or number times entered into the centre zone (Fig 3.13 B). Like the EPM apparatus, the white OF apparatus was not conducive to tracking the albino A/J males.

Further measures were obtained such as the amount of time spent in the periphery zone, number of entries into the periphery zone, total distance travelled (cm) and mean

velocity of the animal (cm/s). No significant differences were found between C57BL/6J^{WT} and C57BL/6J^{PAIS} mice for time spent in the periphery zone (Fig 3.14 A) or number of entries into the periphery zone (Fig 3.14 B). When total distance (cm) and mean velocity (cm/s) was compared there were no significant differences in either measure (Fig 3.15 A & B).

3.5.3.3 Light/Dark Box

(A) Manual Scoring

Mice were compared for the amount of time spent in the light area of the light/dark box (LDB). A/J male mice did not differ significantly in the amount of time spent in the light area compared to C57BL/6J^{WT} and C57BL/6J^{PAIS} mice (Fig 3.16 A). C57BL/6J^{PAIS} mice did not differ from C57BL/6J^{WT} on amount of time spent in the light area (Fig 3.16 A).

A/J mice entered into the light area significantly fewer times compared to C57BL/6J^{WT}, but did not differ from C57BL/6J^{PAIS} (Fig 3.16 B). There was no significant difference between C57BL/6J^{WT} and C57BL/6J^{PAIS} males for the number of entries into the light area (Fig 3.16 B). Fecal boli were collected and counted after testing as an extra measure of anxiety. No significant differences in number of fecal boli were found between the C57BL/6J^{WT} and C57BL/6J^{PAIS} groups (Fig 3.17). However, the A/J mice had significantly more boli after the trial when compared to both C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice (Fig 3.17).

3.5.4 Social Interaction Open Field Test

Time in the interaction zone was compared for Trial A (empty zone) vs Trial B (zone with novel mouse) between C57BL/6J^{WT} and C57BL/6J^{PAIS} mice. There were no

differences in the amount of time spent the interaction zone, or the frequency entered into the interaction zone, across Trials A vs B for either C57BL/6J^{WT} or C57BL/6J^{PAIS} males (Table 3.5). Similarly, there was no significant difference in the number of head points towards the cage within the interaction zone in Trial A or B between phenotype groups. The results suggest no difference in social interaction traits between C57BL/6J^{WT} and C57BL/6J^{PAIS} males.

Figure 3.1: External Anatomy of C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice

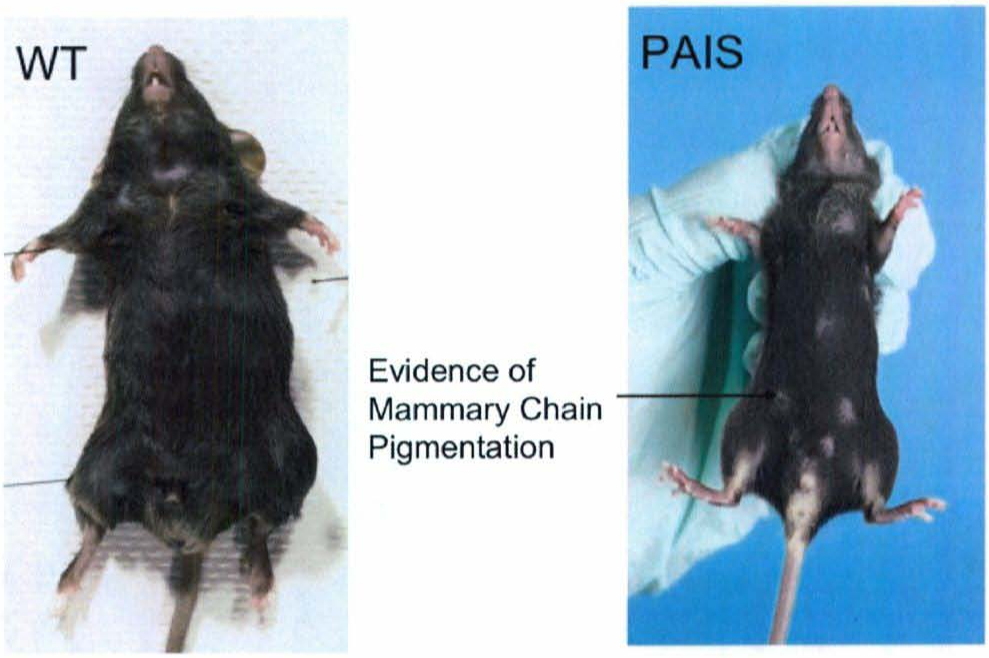


Figure 3.1 shows the external anatomy of both C57BL/6J^{WT} and C57BL/6J^{PAIS} mice. The C57BL/6J^{PAIS} male shows evidence of mammary chain pigmentation, a visibly reduced anal-genital distance, and lack of a scrotum. This is a consistent representative of the external anatomy of both C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice.

Table 3.1: Body Weights and Anal-Genital Distances of C57BL/6J^{WT}, C57BL/6J^{PAIS} male mice versus C57BL/6J^{WT} female mice at 50 d of age.

	Genotype	Body wt. (g)	Anal-Genital Distance (mm)
C57BL/6J^{WT}	Male (n=14)	22.35 ± 1.5	19.14 ± 2.1
C57BL/6J^{PAIS}	Male (n=14)	22.75 ± 2.3*	12.11 ± 0.8*, ₸
C57BL/6J^{WT}	Female (n=14)	17.47 ± 0.8	7.8 ± 0.7

(mean ± sd, *, ₸ p<0.0001, one-way anova)

Table 3.1 shows the body weights and anal-genital distances of the C57BL/6J^{WT} male, C57BL/6J^{PAIS} male, and C57BL/6J^{WT} female mice, n=14 for all three groups. C57BL/6J^{PAIS} males were intermediate for anal-genital distance. They had a significantly smaller distance when compared to C57BL/6J^{WT} males (₸), and a significantly larger distance when compared to C57BL/6J^{WT} females (*) [$F(2, 39) = 225.5$, $p < 0.0001$]. C57BL/6J^{PAIS} and C57BL/6J^{WT} male mice did not differ in body weight, however C57BL/6J^{PAIS} male mice were significantly heavier than C57BL/6J^{WT} females (*) [$F(2, 39) = 44.90$, $p < 0.0001$]. The (*) represents the comparison between C57BL/6J^{PAIS} males and C57BL/6J^{WT} females, and the (₸) represents the comparison between C57BL/6J^{PAIS} and C57BL/6J^{WT} males.

Figure 3.2: Internal Anatomy of C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice

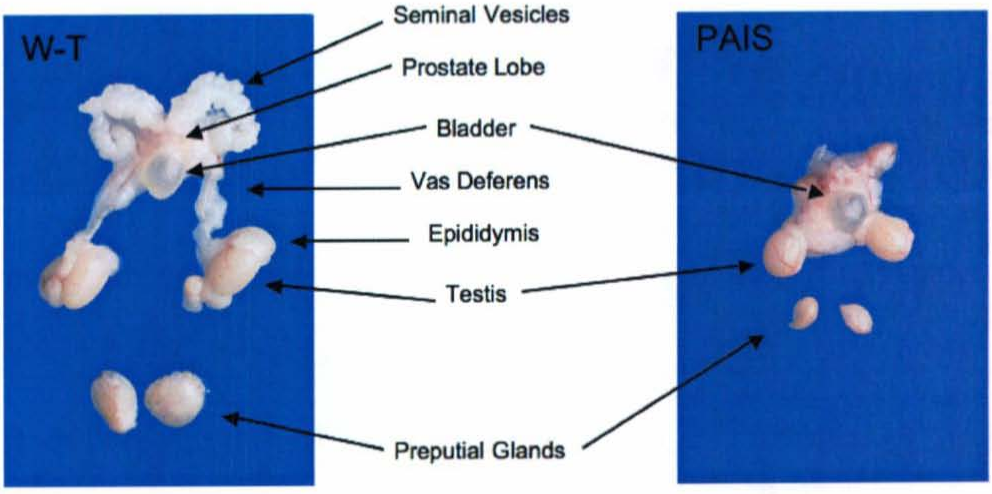


Figure 3.2 shows the internal reproductive structures of the PAIS mutant mouse compared to a normal WT male mouse. The mice harboring the PAIS mutation are missing the seminal vesicles, prostate lobe, vas deferens, and the epididymis. This is representative internal anatomy of C57BL/6J^{WT} and C57BL/6J^{PAIS} mice.

Table 3.2: Organ and body Weights (testes, kidneys, preputial glands) for C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice at 10-12 wks.

	Age (wks)	Testes pr.wt./body (mg/g)	Kidney pr.wt./body (mg/g)	Preputial Gland pr.wt./body (mg/g)	Body Weight (g)
C57BL/6J^{WT} (n=18)	10-12	7.77 ± 0.64	13.69 ± 0.92	2.58 ± 0.63	28.13 ± 2.02
C57BL/6J^{PAIS} (n=18)	10-12	2.97 ± 1.10**	12.45 ± 1.01*	1.31 ± 0.23**	29.05 ± 3.75

mean ± sd, **p<0.0001, *p<0.05, paired t-test)

Table 3.2 shows the pair weights of the testes, kidneys, and preputial glands of C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice at 10-12 wks, normalized to body weight. These males were used in the male typical behavioural tests, thus they are littermate pairs and a paired t-test is used. All three of the androgen responsive organs differed significantly between groups, n=18. The testes were significantly smaller in the C57BL/6J^{PAIS} group when compared to the C57BL/6J^{WT} group, $t(17) = 14.70$, $p < 0.0001$. The same was true for the kidneys, $t(17) = 4.004$, $p = 0.0009$, and for the preputial glands, $t(17) = 8.417$, $p < 0.0001$. There was no significant difference between groups in body weight.

Figure 3.3: Genotyping Assay for Sex Chromosomes

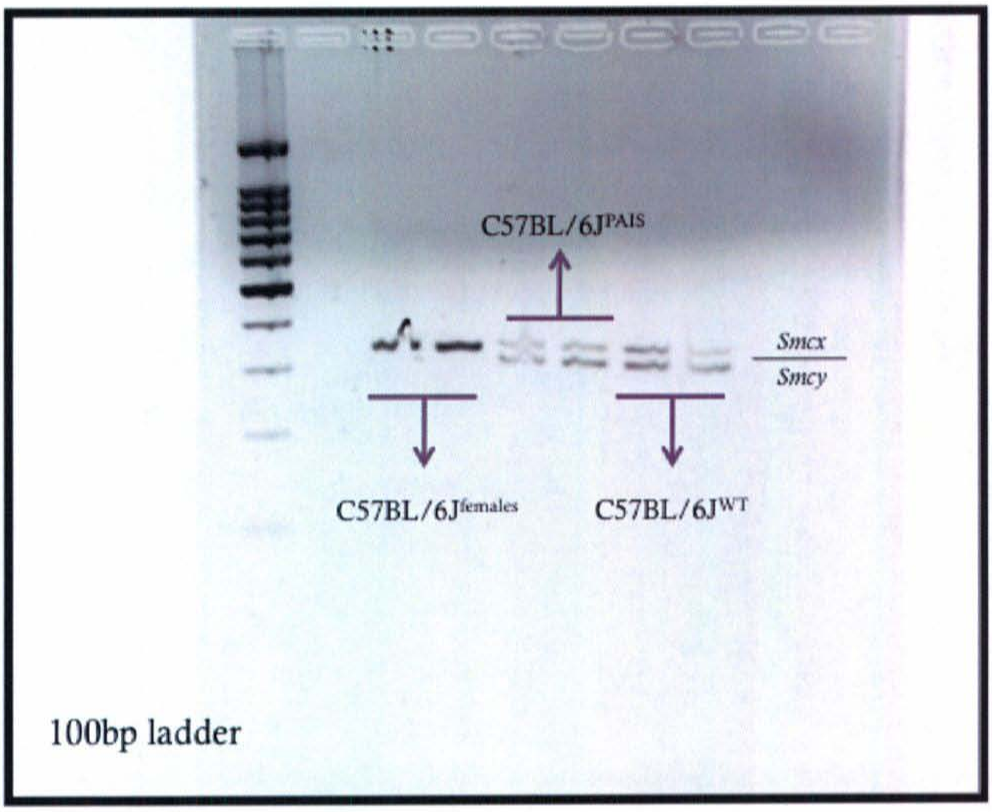


Figure 3.3 shows the results of the genotyping assay for sex chromosomes. The presence of the *Smcy* and *Smcx* genes as found in the PAIS and WT groups is typical of XY males.

Table 3.3: Results of serum assay for LH, FSH, and T in 30 d and 50 d C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice.

Phenotype	Age Group	LH ng/ml	FSH ng/ml	T ng/dl
C57BL/6J^{WT}	30 Day	0.794 ± 1.328 (n=10)	42.67 ± 6.706 (n=10)	65.51 ± 74.01 (n=10)
C57BL/6J^{PAIS}	30 Day	4.385 ± 7.847 (n=10)	58.38 ± 9.488* (n=9)	38.79 ± 40.70 (n=10)
C57BL/6J^{WT}	50 Day	0.258 ± 0.424 (n=6)	34.61 ± 10.69 (n=16)	53.65 ± 54.26 (n=15)
C57BL/6J^{PAIS}	50 Day	3.595 ± 2.242* (n=6)	51.26 ± 11.94* (n=17)	74.41 ± 74.45 (n=17)

(mean ± sd, *p<0.05, unpaired t-test)

Table 3.3 shows the results from the serum assay for LH, FSH, and T at both 30 d and 50 d. When C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice were compared for LH, there was no significant difference at 30 d, $t(18) = 1.427$, $p = 0.1707$; however at 50 d C57BL/6J^{PAIS} mice had significantly higher levels of LH, $t(10) = 3.582$, $p = 0.0050$. When phenotypes were compared for FSH, C57BL/6J^{PAIS} males had significantly higher levels at 30 d, $t(17) = 4.202$, $p = 0.0006$. They also had significantly higher levels at 50 d, $t(31) = 4.213$, $p = 0.0002$. When T was examined there was no significant difference at 30 d, $t(18) = 1.000$, $p = 0.3304$, or at 50 d, $t(30) = 0.8907$, $p = 0.3802$.

Table 3.4: Organ Weights from the Androgen Sensitivity Test and % change with treatment.

(a)

	Seminal Vesicles pr.wt/ body (mg/g)	Kidney pr.wt/ body (mg/g)	Preputial Gland pr.wt./ body (mg/g)
Testosterone WT	6.15 ± 0.33	15.27 ± 0.99	2.39 ± 0.51
Testosterone PAIS	-	14.03 ± 1.10	1.21 ± 0.33**
Empty WT	1.37 ± 0.35*	12.32 ± 0.98	1.31 ± 0.35
Empty PAIS	-	12.17 ± 1.73	0.89 ± 0.21**

Column 1 (mean ± sd, *p<0.0001, unpaired t-test)
Columns 2-3(mean ± sd, **p<0.05, *p<0.0001, 2 x 2 ANOVA)

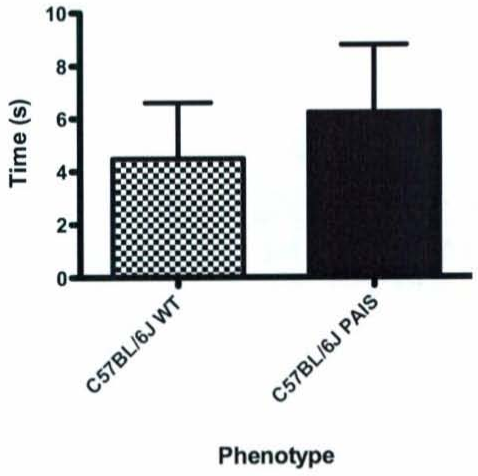
(b)

	Seminal Vesicles % change	Kidneys % change	Preputial Glands % change
Testosterone WT	448.9%	130.8%	185.3%
Testosterone PAIS	-	117.2%	155.1%

Table 3.4 (a) shows the organ weights from the androgen sensitivity test for all four groups: T WT, T PAIS, Empty WT, and Empty PAIS. Used as a positive control, the seminal vesicles were significantly smaller for WT with the empty capsule, $t(15) = 10.04$, $p < 0.0001$. There was no significant effect of phenotype x capsule treatment when the kidneys were compared between groups, $F(30,1) = 1.686$, $p = 0.2040$. There was however, a significant effect on phenotype x capsule treatment when the preputial glands were examined $F(29, 1) = 8.539$, $p = 0.0067$. Table 3.3 (b) indicates the % change that the T treatment produced on each androgen responsive organ of both WT and T PAIS groups.

Figure 3.4: Comparison of Aggressive Behaviour in C57BL/6J^{WT} and C57BL/6J^{PAIS} males using the Resident-Intruder Test

(A)



(B)

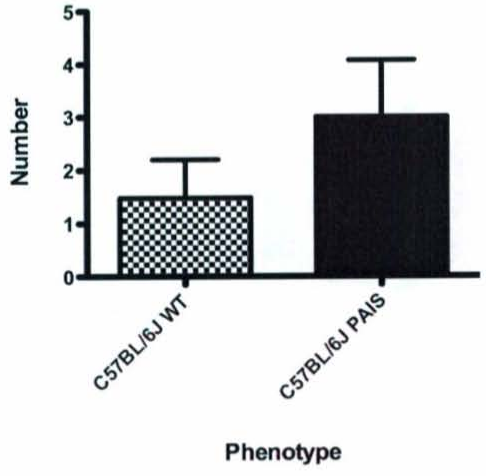
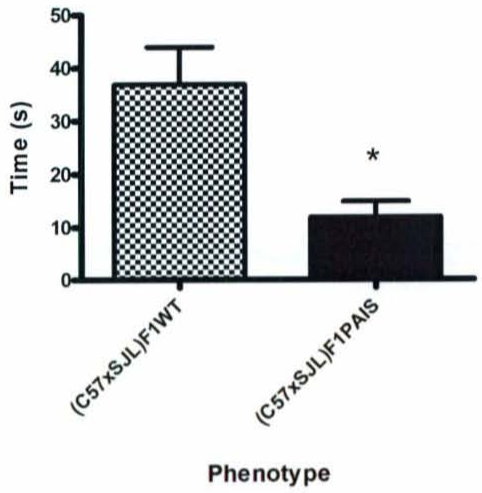


Figure 3.4 shows the time spent fighting (A) and the number of fights (B) during the resident intruder test of aggression using manual scoring. C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice did not significantly differ in time spent fighting, $t(24) = 0.6086$, $p = 0.5485$, or in the frequency of fights, $t(24) = 1.412$, $p = 0.1681$, n=25 pairs.

Figure 3.5: Comparison of aggressive behaviour with a (C57xSJL)F1 hybrid background using the resident-intruder test

(A)



(B)

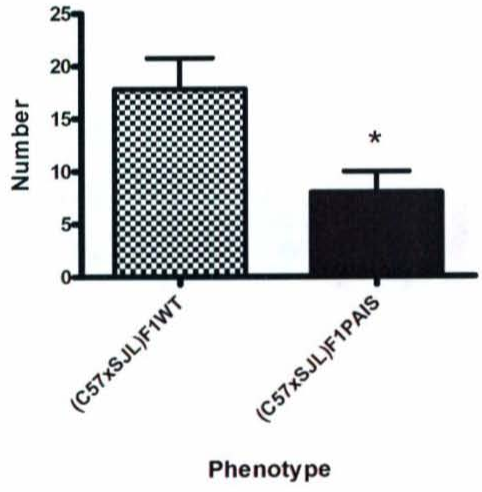
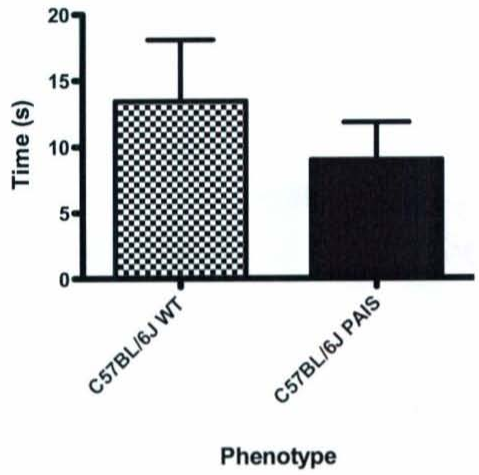


Figure 3.5 shows the time spent fighting (A) and the number of fights (B) during the resident intruder test using manual scoring. (C57xSJL)F1^{WT} were significantly more aggressive on both measures; they fought more often, $t(18) = 2.769$, $p = 0.0127$, and for a longer duration, $t(18) = 3.272$, $p = 0.0042$, when compared to (C57xSJL)F1^{PAIS} males, n=10 pairs.

Figure 3.6: Comparison of Sexual Behaviour using the Simple Mounting Test

(A)



(B)

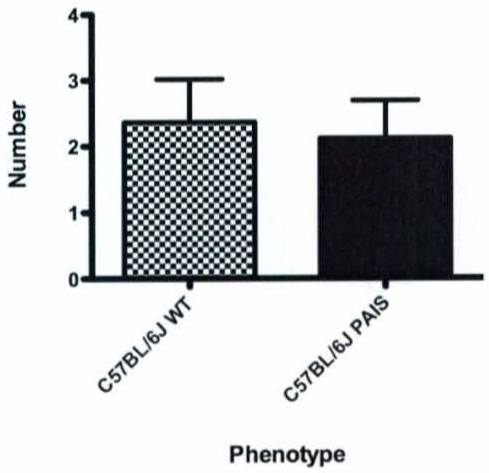
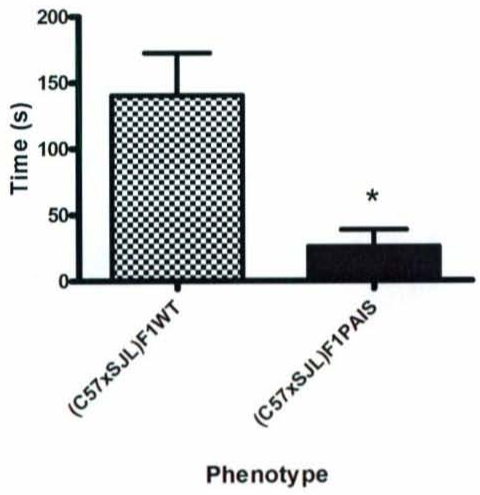


Figure 3.6 shows the time spent mounting (A) and the number of mounts (B) during the simple mounting test of sexual interest using manual scoring. C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice did not significantly differ in frequency, $t(24) = 0.2649$, $p = 0.7943$, or in time spent mounting, $t(24) = 0.8002$, $p = 0.4315$, n=25 pairs.

Figure 3.7: Comparison of Sexual Behaviour with a (C57xSJL)F1 hybrid background using the Simple Mounting Test

(A)



(B)

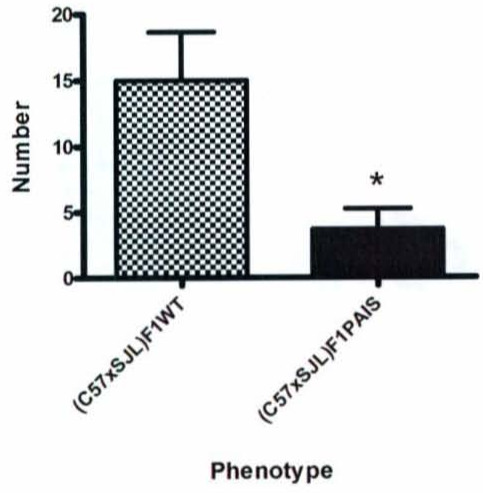
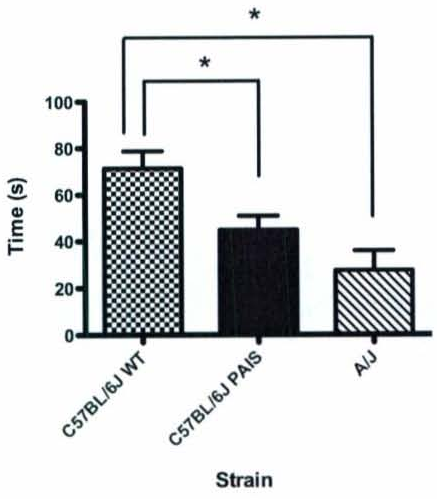


Figure 3.7 shows the time spent mounting (A) and the number of mounts (B) during the sexual interest test using manual scoring. (C57xSJL)F1^{WT} were significantly more sexually interested on both measures, mounting more often, $t(18) = 2.841$, $p = 0.0108$, and for a longer duration, $t(18) = 3.322$, $p = 0.0038$, when compared to (C57xSJL)F1^{PAIS} males, n=10 pairs.

Figure 3.8: Comparison of Anxiety-like Behaviour using the Open Arms of the Elevated Plus Maze with manual scoring

(A)



(B)

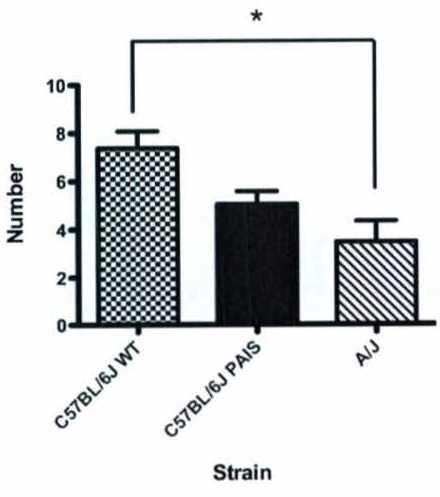
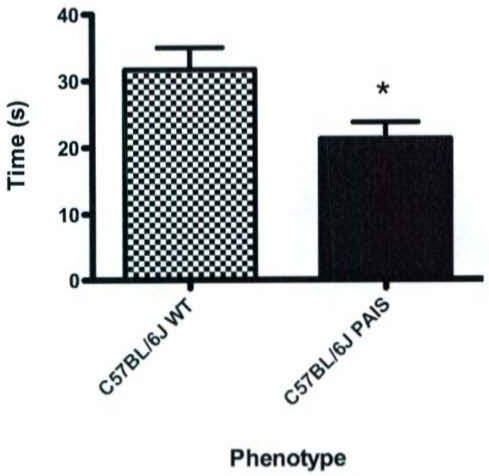


Figure 3.8 shows the time spent in the open arms (A) and the number of entries into the open arms (B) of the elevated plus maze using manual scoring. C57BL/6J^{WT} spent significantly more time in the open arms than both the C57BL/6J^{PAIS} and A/J males, $F(2, 58) = 9.149$, $p = 0.0004$. C57BL/6J^{WT} males also entered into the open arms significantly more than A/J males, $F(2, 58) = 7.789$, $p = 0.0010$, n=19-21 pairs. Post-hoc comparisons were made using Tukey's multiple comparison test.

Figure 3.9: Comparison of Anxiety-like Behaviour using the Open Arms of Elevated Plus Maze with Ethovision

(A)



(B)

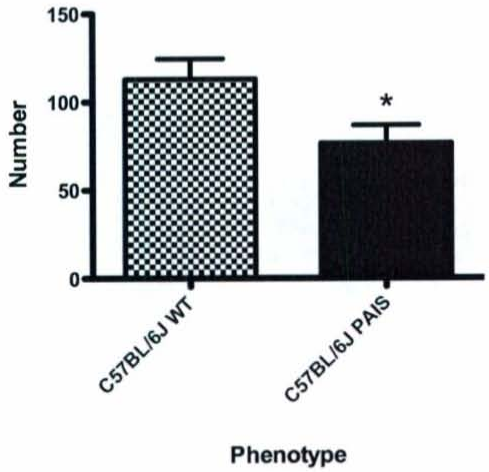
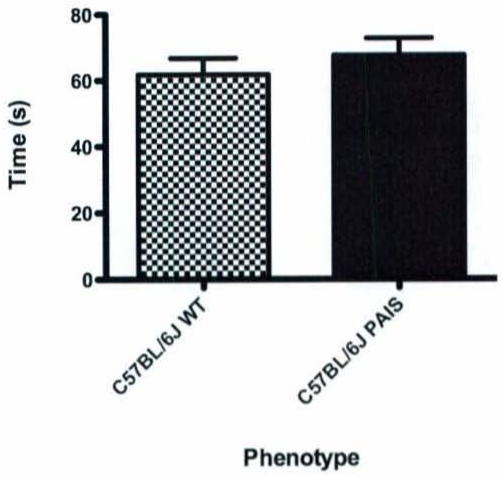


Figure 3.9 shows the time spent in the open arms (A) and the number of entries into the open arms (B) using computerized scoring. C57BL/6J^{WT} spent significantly more time, $t(20) = 2.363$, $p = 0.0284$, and entered into the open arms significantly more often than the C57BL/6J^{PAIS} male mice, $t(20) = 2.128$, $p = 0.0460$, n=21 pairs.

Figure 3.10: Comparison of Anxiety-like Behaviour using the Closed Arms of Elevated Plus Maze with Ethovision

(A)



(B)

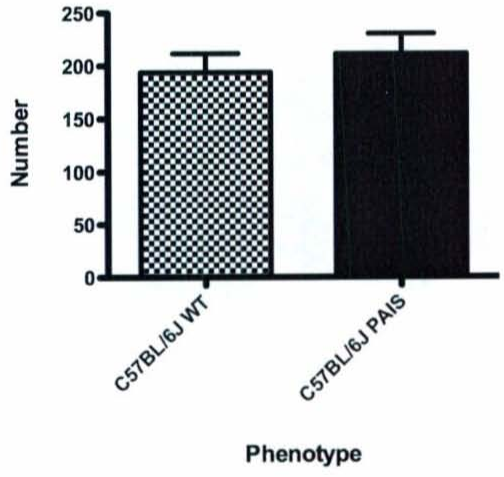
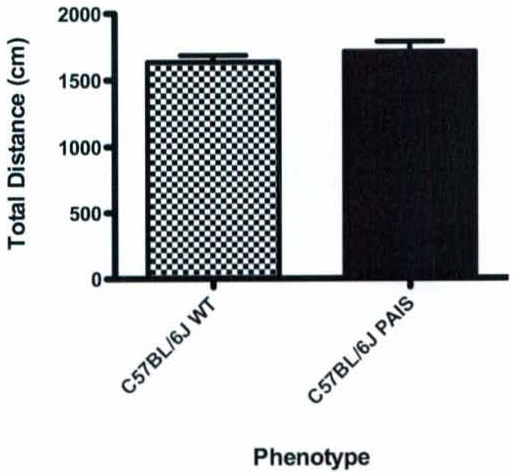


Figure 3.10 shows the time spent in the closed arms (A) and the number of entries into the closed arms (B) using computerized scoring. There was no significant difference between C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice on measures of time, $t(20) = 0.9241$, $p = 0.3665$, or frequency, $t(20) = 0.8054$, $p = 0.4301$, n=21 pairs.

Figure 3.11: Comparison of Locomotor Behaviours in the Elevated Plus Maze using Ethovision

(A)



(B)

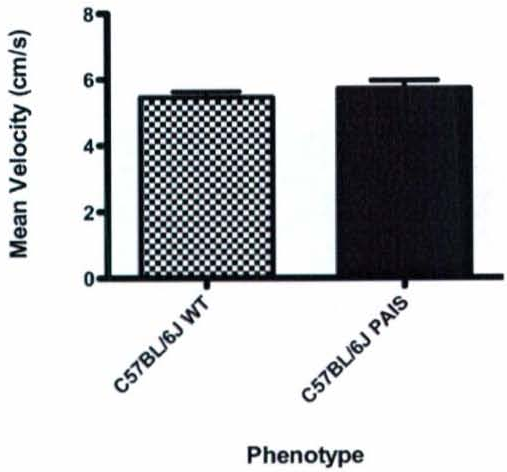
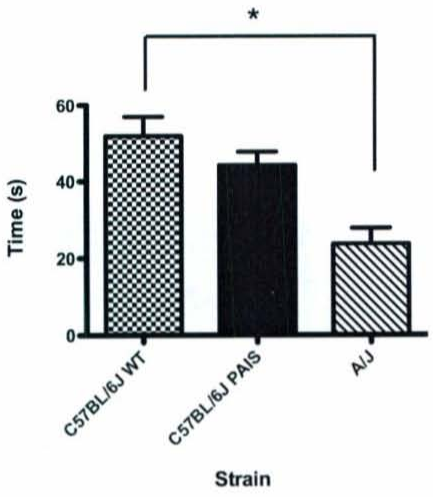


Figure 3.11 shows the total distance travelled (A) and the mean velocity (B) of C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice in the elevated plus maze using computerized scoring. There was no significant difference between the C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice in distance travelled, $t(20) = 0.8029$, $p = 0.4315$, or in mean velocity, $t(20) = 0.7640$, $p = 0.4358$, n=21 pairs.

Figure 3.12: Comparison of Anxiety-like Behaviours in the Open Field using manual scoring

(A)



(B)

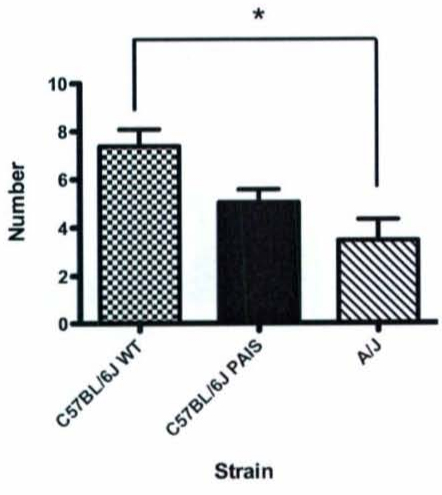
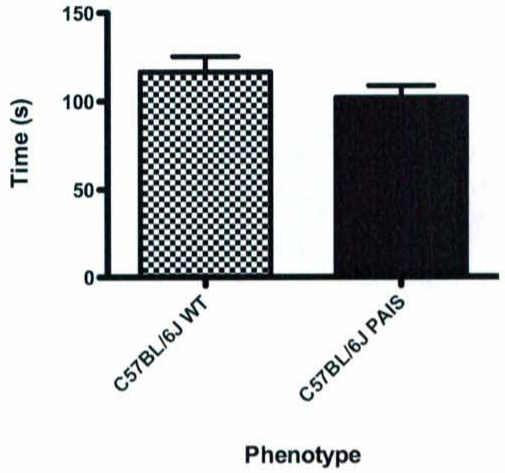


Figure 3.12 shows the time spent in the centre zone (A) and the number of entries into the centre zone (B) of the open field using manual scoring. C57BL/6J^{WT} spent significantly more time in the centre zone, $F(2,55) = 10.31$, $p = 0.0002$, and entered into the centre zone significantly more often than A/J males, $F(2,55) = 71.04$, $p < 0.0001$, $p<0.05$, n=19-21 pairs. Post-hoc comparisons were made using Tukey's multiple comparison test.

Figure 3.13: Comparison of Anxiety-like Behaviour using the Centre Zone of the Open Field with Ethovision

(A)



(B)

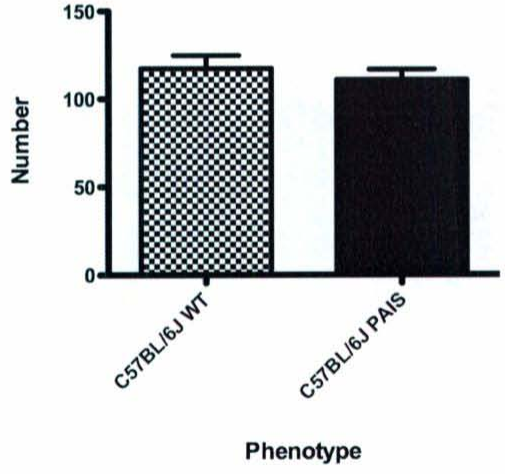
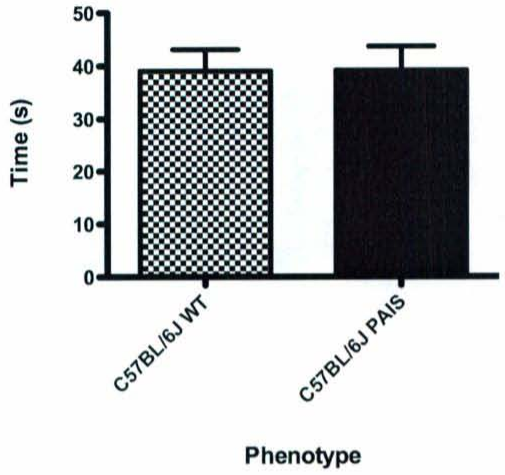


Figure 3.13 shows the time spent in the centre zone (A) and the number of entries into the centre zone (B) of the open field using computerized scoring. C57BL/6J^{WT} did not differ significantly on time, $t(20) = 1.350$, $p = 0.1922$, or number of entries into the centre zone from C57BL/6J^{PAIS} male mice, $t(20) = 0.8620$, $p = 0.3989$, n=21 pairs.

Figure 3.14: Comparison of Anxiety-like Behaviour using the Periphery Zone of the Open Field with Ethovision

(A)



(B)

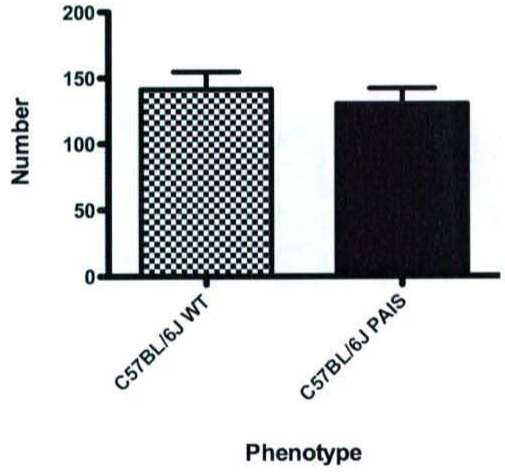
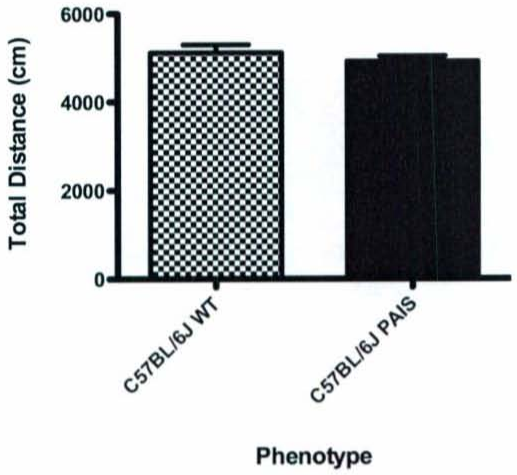


Figure 3.14 shows the time spent in the periphery zone (A) and the number of entries into the periphery zone (B) of the open field using computerized scoring. C57BL/6J^{WT} did not differ significantly on time, $t(20) = 0.03171$, $p = 0.9750$, or number of entries into the periphery, $t(20) = 0.6376$, $p = 0.5310$ zone from C57BL/6J^{PAIS} male mice, n=21 pairs.

Figure 3.15: Comparison of Locomotor Behaviours in the Open Field using Ethovision

(A)



(B)

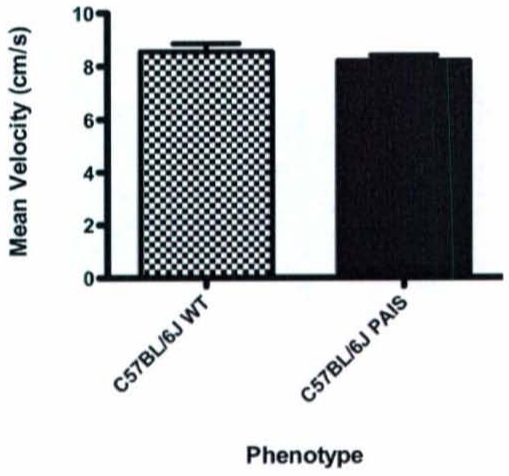
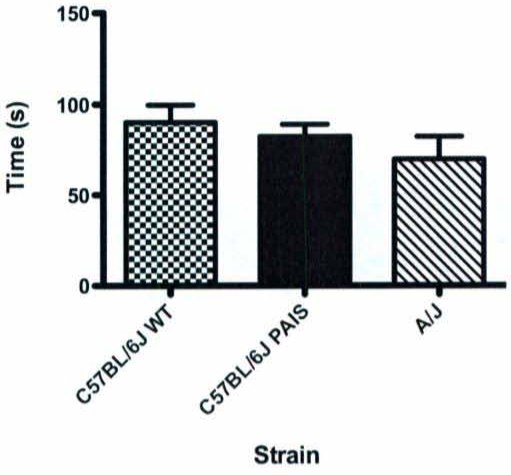


Figure 3.15 shows the total distance travelled (A) and the mean velocity (B) of C57BL/6J^{WT} and C57BL/6J^{PAIS} male mice in the open field using computerized scoring. There was no significant difference between the C57BL/6J^{WT} and C57BL/6J^{PAIS} mice on total distance travelled, $t(20) = 0.9410$, $p = 0.3579$, or on mean velocity, $t(20) = 0.9811$, $p = 0.3383$, n=21 pairs.

Figure 3.16: Comparison of Anxiety-like Behaviours in the Light/Dark Box using manual scoring

(A)



(B)

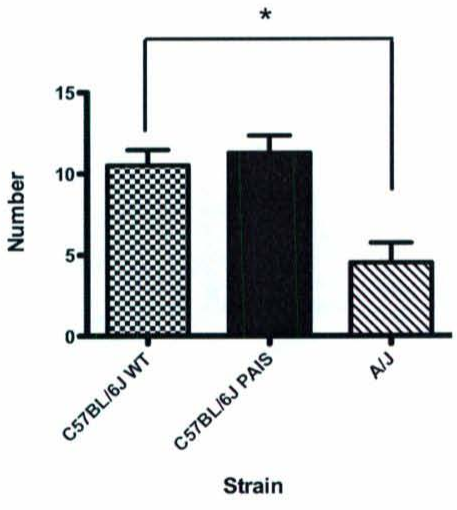


Figure 3.16 shows the time spent in the light area (A) and the number of entries into the light area (B) of the light/dark box using manual scoring. C57BL/6J^{WT} did not differ significantly from A/J or C57BL/6J^{PAIS} male mice on the measure of time, $F(2, 56) = 1.105$, $p = 0.3382$. A/J male mice entered into the light area significantly fewer times than C57BL/6J^{WT} males, $F(2, 56) = 11.19$, $p<0.0001$, $p<0.05$, n=19-21 pairs. Post-hoc comparisons were made using Tukey's multiple comparison test.

Figure 3.17: Fecal Boli Counts following a trial in the Light/Dark Box

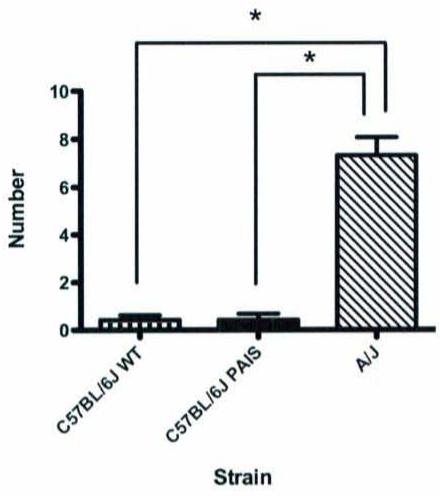


Figure 3.17 shows the number of fecal boli collected following testing in the light/dark box for C57BL/6J^{WT}, C57BL/6J^{PAIS} and A/J male mice. Significantly higher numbers of boli were found from A/J male mice compared to C57BL/6J^{WT} and C57BL/6J^{PAIS} males, $F(2, 58) = 76.77$, $p<0.0001$, $p<0.05$, n=19-21 pairs. Post-hoc comparisons were made using Tukey's multiple comparison test.

Table 3.5: Comparison of social behaviours, using the social interaction open field test, between C57BL/6J^{WT} and C57BL/6J^{PAIS} males

Measure	Trial	C57BL/6J^{WT}	C57BL/6J^{PAIS}
Time in Interaction Zone	A	51.04 ± 11.34	59.04 ± 13.73
Time in Interaction Zone	B	38.41 ± 15.44	44.65 ± 18.05
Number of Entries into Interaction Zone	A	70.95 ± 16.48	81.05 ± 22.54
Number of Entries into Interaction Zone	B	65.48 ± 26.65	81.67 ± 37.09
Time Head Pointing	A	11.00 ± 4.51	13.54 ± 3.93
Time Head Pointing	B	10.32 ± 7.49	9.77 ± 7.49
Number of Head Points	A	57.38 ± 17.46	65.00 ± 22.74
Number of Head Points	B	44.38 ± 19.70	51.71 ± 26.97

(mean ± sd, 2 x 2 ANOVA)

Table 3.5 shows the results from the social interaction open field test. In Trial A no stimulus animal was present, and in Trial B a stimulus animal was present. There was no effect of phenotype x trial for the time spent in the interaction zone, $F(80, 1) = 0.07389$, $p = 0.2497$, nor for the frequency entered into the interaction zone, $F(80,1) = 0.2723$, $p = 0.6032$. When head pointing towards the interaction zone was measured there were also no effects of phenotype x trial for time, $F(80,1) = 1.345$, $p = 0.2497$, or for frequency, $F(80,1) = 0.0008847$, $p = 0.9763$, n=21 pairs.

Chapter 4 – DISCUSSION

4.1 A Mouse Model for PAIS resembling Reifenstein Syndrome

Partial AIS (PAIS) lies in the middle of the AIS spectrum, clinically described as pseudohermaphroditism, or Reifenstein syndrome; it shows a greater degree of variation in gender presentation than CAIS. The PAIS mouse mutation described in this work is X-linked and maternally transmitted, which is strongly suggestive of a mutation in the mouse *Ar* gene on Chr X similar to *Tfm* mouse models of CAIS.

Anatomical differences were observed in the C57BL/6J^{PAIS} mice when compared against C57BL/6J^{WT} control male mice at all post-natal ages examined. The C57BL/6J^{PAIS} males are intermediate between genders, not quite male and not quite female. Externally, they have evidence of mammary chain pigmentation as well as a significantly shorter anal-genital distance. Internally, they are comparable to human Reifenstein patients, missing the seminal vesicles, prostate lobes, vas deferens, and the epididymis. Furthermore, they have undescended which is characteristic of Reifenstein patients. Unlike the variation observed among humans with PAIS, the phenotype is very consistent among C57BL/6J^{PAIS} male mice, and this is a great platform for investigating health outcomes of PAIS.

Androgen responsive organs of PAIS mice were smaller than those of WT mice. Results of the androgen sensitivity test showed that even when exogenous T was supplied, the preputial glands in C57BL/6J^{PAIS} males did not respond as much as in WT males, suggesting a failure of androgen response. Comparing organ responses between sham and T-treated C57BL/6J^{PAIS} males, the significant degree of organ mass incresase

with T exposure confirms that partial androgen insensitivity is anaccurate descriptor for C57BL/6J^{PAIS} mutant males. In contrast, a complete loss of androgenic signalling is observed in *Tfm* male mice (Lyon & Hawkes, 1970).

AIS initiates a change in HPG axis regulation due to a loss of negative feedback. Humans with Reifenstein Syndrome differ endocrinologically from normal males, having significantly increased levels of LH and T; FSH levels range from normal to high (Campo et al., 1979). In *Tfm* male mice, levels of T are similar between *Tfm* and WT mice up until about post natal day 10, after which time the *Tfm* males have significantly reduced serum T (Goldstein & Wilson, 1972). Serum gonadotropin levels (FSH/LH) are also elevated in *Tfm* mice (Lyon & Hawkes, 1970; Murphy et al., 1994). In contrast, no differences in T levels between C57BL/6J^{WT} and C57BL/6J^{PAIS} mice were found at either 30 d or 50 d (Table 4.1). A large degree of variability was noted for serum T between individuals in both groups, even though serum collections were consistently performed in the morning to reduce variation caused by circadian rhythms, stored at -20 °C, and thawed only once prior to assay. A large degree of serum T variation has been noted in previous reports using pooled serum samples, suggesting a larger population sample is required when analyzing individual serum samples (Selmanoff et al., 1977).

LH data showed a peak at 30 d for C57BL/6J^{PAIS} although not significantly different, however at 50 d C57BL/6J^{PAIS} had significantly elevated LH levels compared to C57BL/6J^{WT} males (Table 4.1). FSH data showed that serum FSH is significantly elevated in C57BL/6J^{PAIS} males at 30 d and 50 d time points. These results could be attributed to a failure in feedback within the hypothalamic-pituitary-axis.

Table 4.1: Degree of function and endocrinology of PAIS, *Tfm*, ARKO mice, and *Tfm* rat compared to WT males.

	Degree of AR function	LH serum levels	FSH serum levels	T serum levels	References
Mouse *Tfm*	null	J: *Tfm* > WT A: *Tfm* > WT	A: *Tfm*> WT	J: *Tfm* = WT A: *Tfm* < WT	Lyon & Hawkes, 1970 Goldstein *et al.*, 1972 Murphy *et al.*, 1994 Jones *et al.*, 2003
Mouse ARKO	null	N/A	N/A	A: ARKO < WT	Notini, *et al.*, 2005
Rat *Tfm*	10-15% Ligand Binding Activity	J: *Tfm* > WT A: *Tfm* > WT	A: No Difference or *Tfm* > WT	J: *Tfm* > WT A: *Tfm* > WT	Purvis *et al.*, 1977 Naess *et al.*, 1976 Yarborough *et al.*, 1990
Mouse PAIS (C57BL/6J)	Partial	J: PAIS = WT A: PAIS > WT	J: PAIS > WT A: PAIS > WT	J: PAIS = WT A: PAIS = WT	

Table 4.1 shows the degree of function for PAIS, *Tfm,* ARKO mice, and *Tfm* rat compared to WT males. Both the mouse *Tfm* and ARKO have null AR function, rat *Tfm* has 10-15% binding activity of T to AR, and the PAIS mouse has partial functioning. Adult (A) mice are approximately 6wks and rats are older than 3 months. Juvenile mice (J) are around 3-6 wks of age and rats are between 2 and 3 months. LH levels in the adult and juvenile *Tfm* mouse are significantly greater compared to WT males. FSH is also significantly elevated in adult *Tfm* mice, and T is significantly decreased in adult *Tfm* mice. Mouse ARKO males have significantly greater levels of T during adulthood. Rat *Tfm* males have significantly higher levels of LH and T during adulthood and as juveniles. The PAIS mutation did not have an effect on T levels of adults or juveniles nor did it have an effect on juveniles for LH. PAIS males had significantly greater LH levels as adults. In both adults and juveniles serum FSH was significantly elevated in PAIS males. N/A indicates that this measure has not been assessed.

4.2 The PAIS mutation alters Male-Typical behaviours

Androgens exert their effects on males in two different forms: organizational and activational (Phoenix et al., 1959; Morris et al., 2004). Androgens have the ability to exert differential effects on aspects of both physiology and behaviour depending on the time of exposure during development (Phoenix et al., 1959). During embryonic development, the organizational effects of androgens are required during a critical period in order to contribute to the sex-specific development of many organs. The activational effects of androgens in the mature male aid in the masculinization of many behavioural changes seen in animal models (Morris et al., 2004).

In our PAIS model, there are severe effects on the development of the genital tract and this congenital mutation likely has a significant effect on androgen-mediated organizational and activational effects in the brain of PAIS males. The PAIS mutation significantly reduced both types of male-typical behaviours (aggression and sexual interest) in (C57xSJL)F1^{PAIS} hybrid males. This sensitized genetic background supported a greater number of behavioural events and served as a better assay than the C57BL/6J background in which a very low number of behavioural events occurred in both tests. Male typical behaviours such as aggression can vary among inbred background strains (Guillot & Chapouthier, 1996) (Table 4.2). Intermale aggression was tested among 10 inbred strains of mice in order to evaluate strain differences. It was found that C57BL/6 males had the lowest proportion of attacking males of the group and as such, this inbred strain is not as useful as certain others for determining (especially decreases of) behavioural aggression. Recently, it was found that males lacking *Ar* in the nervous

system have the ability to initiate masculine behaviours (sexual and territorial) indicating that *Ar* in the brain is not necessary for these displays, but may act to enhance these behavioural phenotypes in males (Juntti et al., 2010). The Juntti group's experiment also used a C57BL/6 genetic background for the ARloxP/Y; Nes-Cre males, and perhaps using C57BL/6 to evaluate sexual behaviour is not the best choice according to our observations. These males exhibited fewer mounts and intromissions when compared to the control males; they also attack intruders for a shorter duration, there is significantly more time in between fights, and there is less overall fighting when compared to the control males (Juntti et al., 2010). Although the *Ar* is not necessary in the ability to initiate masculine behaviours, it enhances this behavioural phenotype in the C57BL/6J^{WT} males.

Table 4.2: Comparison of male-typical behaviours, anxiety-like behaviours and social interactions across PAIS , *Tfm*, ARKO mice, and Rat *Tfm*.

	Strain	Male-Typical Aggression	Male-Typical Sexual Behaviour	Anxiety	Social Interactions	References
Mouse *Tfm*	C57BL/6	*Tfm* < WT	*Tfm* < WT	*Tfm* >WT	N/A	Ono *et al.*, 1974 Rizk *et al.*, 2005 Zuloaga *et al.*, 2008
Mouse ARKO	(C57BL/6 x CD-1)	ARKO < WT	ARKO < WT	N/A	N/A	Sato *et al.*, 2004
Rat *Tfm*	Stanley-Gumbreck	*Tfm* < WT	*Tfm* < WT	N/A	N/A	Meaney *et al.*, 1983 Beach *et al.*, 1977
Mouse PAIS	C57BL/6J	PAIS = WT	PAIS = WT	PAIS > WT	PAIS = WT	
Mouse PAIS	(C57xSJL)F1	PAIS < WT	PAIS < WT	N/A	N/A	

Table 4.2 shows behavioural results in male-typical aggression, sexual behaviour, anxiety, and social interactions in mouse *Tfm*, ARKO mice, rat *Tfm,* and PAIS mice. C57BL/6J^{PAIS} males did not differ from C57BL/6J^{WT} males in tests of male-typical behaviours or social interactions, however were significantly more anxious on the EPM. (C57xSJL)F1^{PAIS} males had reduced male-typical aggression as well as sexual interest when compared with (C57xSJL)F1^{WT}males. N/A indicates that this measure has not been assessed.

4.3 Social Behaviours are not affected by the PAIS mutation

Social interaction behavioural tests have been used in many rodent models of autism. One of the characteristic features of autism is the lack of correct social conduct and inability to socialize normally (Crawley, 2007). In one familiar paradigm designed to test social approach interactions, a mouse is placed in a large box with two compartments; one has an empty wire cage and the other has a wire cage with a stimulus mouse which is usually an adult C57BL/6J unfamiliar male (Crawley, 2007). C57BL/6J mice have been used to test sociability and have been shown to spend significantly more time examining the cage with the stimulus mouse compared to the one without. This strain also has a tendency for high physical activity and social approach to other animals (Scott, 1942; Moy et al., 2004; Moy et al., 2007).

Unlike what has been described in the literature for C57BL/6 mice during tests of social approach, the C57BL/6J^{PAIS} and C57BL/6J^{WT} males reacted in the same manner, and due to the similarities in the behaviour (although unexpected) the PAIS mutation does not appear to affect the social interactions within the social interaction open field test. This is a novel finding testing an AIS model and social approach. There were no significant differences in the following measures between phenotypes: time spent in the interaction zone between trials, number of entries into the interaction zone between trials, time spent head point towards the interaction zone between trials, and the number of head points between trials (Table 3.5).

One suggestion as to why the animals explored the interaction zone in Trial (A) with no stimulus animal could be that during the first trial the wire mesh cage apparatus where the stimulus animal rests in Trial B is still present (although empty) in Trial A. The

fact that a novel object is present in the first trial may be enough to stimulate the animal's interest to explore the interaction zone. By the time the second Trial (B) is underway the animals could be acclimated to the apparatus. Also, the social-interaction open field test was the fourth behavioural test on four consecutive days; during the previous three days mice were housed with littermate pairs, and undergoing anxiety behavioural testing which may have affected their social behaviour. One suggestion would be to house them individually prior to social interaction testing in order to deprive them socially so they may be more drawn to socialize during the social interaction open field test.

4.4 Anxiety and AIS in Animals

Androgens may modulate anxiety. In aging males there is a natural decline of T (andropause) and one of the symptoms associated with this phenomenon is anxiety (Cooper & Ritchie, 2000; Seidman, 2003). Testosterone replacement therapy reduces symptoms of anxiety and mood in andropausal males, and is often chosen as therapy for the disorder (Burris et al., 1992; Velazquez & Bellabarba Arata, 1998). Animal models of anxiety have been used to further our knowledge of the genetic, neurological and neurochemical mechanisms that contribute to anxiety (Crawley, 1999; Milner & Crabbe, 2008; Ramos, 2008). Experiments on the house mouse (*Mus musculus*) support the link between T and anxiety, showing that normal androgenic signaling significantly lowers anxiety, as reflexive T release (during mating) also leads to reduced anxiety (Aikey et al., 2002).

Although the mechanism was not explored in our experimentation, the HPA axis is a probable cause. Activation of the HPA axis is correlated with increases in anxiety (Lund et al., 2004; Lund et al., 2005); androgens play a role in the HPA axis by reducing

the rise of the stress hormones (adrenocorticotropic hormone (ACTH) and corticosterone) following a stressful situation (Aikey et al., 2002; Lund et al., 2004; Lund et al., 2005). HPA axis activity is controlled in part by a subset of parvocellular neurons in the paraventricular nucleus (PVN) of the hypothalamus; the HPA axis receives anxiety input which causes a secretion of corticotropin-releasing hormone (CRH) from these PVN neurons (Weiser et al., 2008). CRH then stimulates the anterior pituitary to secrete (ACTH) which in turn drives the glucocorticoid production and release by the adrenal cortex (Weiser et al., 2008). Our data suggest that the PAIS mutation confers a genetic predisposition to anxiety-like behaviour, which may be operative in human PAIS patients, and may compound their anxiety due to an obvious gender disorder.

Conditioned and unconditioned animal models of anxiety exist (Canteras, 2008). Conditioned anxiety responses are associated with a context or a neutral cue; this type of test uses Pavlovian conditioning to evoke an anxiety-like response by pairing a foot shock to something such as a tone or light (Canteras, 2008). Our model of anxiety is unconditioned; the paradigm is itself anxiety-evoking and does not require conditioning to a stimulus. When the C57BL/6J^{WT} and C57BL/6J^{PAIS} males were compared across three tests of anxiety (EPM, OF, LDB) there was a significant difference in one, the EPM. In the EPM, C57BL/6J^{PAIS} males were significantly more anxious compared to littermate C57BL/6J^{WT} males (Table 4.2). This test is thought to measure the mouse's natural tendency to explore a new environment versus their tendency to avoid a potentially dangerous situation. The finding of elevated anxiety-like behaviour in PAIS males mimics what has been reported in *Tfm* males (Zuloaga et al., 2008).

So what does it mean when only one of three tests of anxiety is affected by the

mutation? The consistency of assay, specifically in the field of anxiety research in rodents, has been the subject of much debate. Although rodent models do not fully replicate the human disease, there are fundamental signs that can be observed in order to test theories related to human disorders (Rodgers, 1997). In mouse behavioural studies, "laboratory effects" can lead to source errors and affect results depending on the laboratory, experimenter, or even the apparatus (Brown, 2007). Many experiments are now underway which aim to further validate behavioural tests as well as make sure that the apparati in use truly are testing what they are thought to test (Brown, 2007). However, we are confident in our conclusion that the PAIS mutation increases anxiety-like behaviour in the EPM due to our careful design and study power using >20 littermate pairs of C57BL/6J^{WT} and C57BL/6J^{PAIS} males that experienced a similar maternal environment.

Caution should be taken when analyzing behaviour because locomotion is highly correlated with indices of anxiety, and inbred strains differ in locomotor abilities (Milner & Crabbe, 2008). One of the advantages of using the Ethovision XT tracking system was the ability to examine locomotor defects as possible confounding variables. When C57BL/6J^{WT} and C57BL/6J^{PAIS} males were examined for total distance travelled and velocity, there were no differences on either measure. Having the ability to eliminate locomotion as a confounding variable, we can be confident in our conclusion of their anxiety-like behavioural differences. Therefore the finding of elevated anxiety-like behaviours for C57BL/6J^{PAIS} males in the EPM was not caused by the inability to navigate the space.

Use of A/J mice as a positive control strain provided additional confidence in the

paradigm, apparati and the monitoring of behavioural videos. A/J mice score as a "high" anxiety strain based on their behaviour in anxiety testing. Thus, A/J mice underwent the same series of anxiety testing as C57BL/6J^{WT} and C57BL/6J^{PAIS} males. In all three tests of anxiety (EPM, OF, LDB) the A/J males were significantly more anxious on at least one measure when compared to C57BL/6J^{WT} males. Fecal boli were also collected after all three tests, and the A/J mice defecated significantly more boli than did the C57BL/6J^{WT} mice, indicating that even using a crude measure of anxiety, the A/J mice have higher levels of anxiety. Not only did this validate our anxiety-testing paradigm, but it reflected what is reported in the literature. Due to the color of the A/J strain we were unable to track them using computerized tracking and therefore unable to evaluate their locomotor ability; although A/J mice are known to have locomotor defects (Whalsten & Crabbe, 2003). Across inbred strains, the C57BL/6J inbred mouse is around the 'middle of the pack' for anxiety related behaviours, making them an ideal choice to measure either decreases or increases of anxiety-related behaviour (Milner & Crabbe, 2008). Interestingly, one study suggests that in order to cut down on the emotional stress of multiple tests and avoid human error and variation multiple anxiety tests should be conducted in a single apparatus (Ramos, 2008). By combining the EPM, OF, and LDB into one, simultaneously run test, it is proposed that it would be a more reliable measure of anxiety (Ramos, 2008).

4.5 Translation to Human PAIS (Reifenstein Syndrome)

Disorders of sexual development can be devastating to a person or his/her family. Animal models of such disorders are valuable because we are able to broaden our knowledge of the disorder by looking at other aspects of gender disorders such as

anatomical, physiological, and behavioural features. The possibility also exists for generating possible treatments and pharmaceutical aids.

The opinions on disclosure to the patient due to the psychosocial considerations have been varied throughout the years. When Morris (1953) first described AIS based on 82 cases of CAIS he strongly argued that the disorder and actual gender should be kept from the patient and that the physicians should only explain to the patients that they were unable to bear children. This idea of discretion was even carried through to the 90's when Shah stated that "the disclosure of the genotype is irrelevant to care and may be confusing to patient and family" (Shah et al., 1992). Currently, the genotype is revealed as soon as it is discovered at the time of diagnosis; when the diagnosis is made in a child, parents are integrated into psychotherapy to learn how to disclose the information to the child. When the diagnosis is made during adolescence, the patient is informed along with the parents immediately (Laufer, 2005). Money (1984) concluded there was no increased rate of gender identity disorder, bisexuality, or homosexuality in CAIS patients. With an intermediate disorder such as PAIS, the varying degree of masculinity may be more confusing to the patient and the rates for bisexuality, homosexuality, and gender confusion are higher than that of the general population (Meyer-Bahlburg, 1982; Money et al., 1984). Our behavioural assessment of congenital PAIS in mouse model suggests a genetic contribution to anxiety-like behaviour, which may be compounding the psychological outcomes in PAIS patients.

4.6 Future Directions

- Explore the genetic mechanism of the PAIS mutation. Although the mechanism is still unknown, there is evidence that it resides on Chr X, as it is passed on maternally.

Phenotypic mapping has narrowed the list of candidate genes, which includes *Ar*. *Ar* gene sequencing, RNA transcript and protein expression studies will be helpful to determine the molecular genetic cause of PAIS in this strain.

- Incorporate the novel object test into the battery of tests for the PAIS mice so that the anxiety-tests would be directly comparable between PAIS mice and *Tfm* mice.
- Examine the effectiveness of certain anxiolytic drugs in the male PAIS mice in order to further validate the anxiety studies. Having the ability to either reduce or increase the levels of anxiety seen in PAIS mice would serve as an effective tool for further research on the treatment of anxiety in gender disorders, specifically PAIS (Hovatta & Barlow, 2008).
- Future tests could examine spatial memory and the size and structure of hippocampal neurons, the sexually dimorphic nucleus of the hypothalamus and the SNB in the spinal cord.
- Repeat the social interaction testing using a more sensitive protocol. One suggestion would be to house the mice individually prior to the social interaction trials.
- Consider the PAIS mouse model for its potential as an accelerated model for andropause. Naturally, as men age there is a gradual decline of androgens and this occurrence has been variably called: andropause, male menopause, and partial androgen deficiency of aging males (PADAM) (Amore, 2005). This condition is variable in men, and has a wide range of symptoms and signs associated with it such as weakness, fatigue, reduced muscle and bone mass, oligospermia, decline in sexual drive (libido), depression, anxiety, and memory impairment (Wang et al., 1996; Seidman, 2003). It has also been associated with osteoporosis, increase in body fat, erectile dysfunction, and

cognitive deficits including depression (Vermeulen, 1979; Vermeulen & Kaufman, 1995). Declines in libido have been reported in aging men, and studies have shown that in eugonadal men, suppression of T led to a reduced sexual desire as well as reduced frequency for sexual activity (Bagatell et al., 1994). Improvements are observed in some, but not all, men who receive T replacement (Bagatell et al., 1994). One difference to note would be the partial functioning of the *AR* in PAIS males and the very gradual decline of T in andropausal males. Many of the symptoms and signs appear to be the same due to problems with androgen signalling.

- Study the mouse model of PAIS to determine its appropriateness to test and determine health outcomes in human PAIS patients. Due to the fact that it is an intermediate model of disease, yet still maintains a consistent phenotype, this permits the dissection of gene-gene and gene-environment relationships important to Ar signalling. Through our experimentation the PAIS mutation has been crossed onto a different inbred background from C57BL/6J carriers. The SJL/Bm inbred strain was chosen due to their elevated aggression levels in males, and despite the fact that these were hybrids the developmental phenotype (anatomy) was consistent and proved a good example of genetic crosses to introduce another phenotype of interest. The model could be used to examine the impact of the PAIS mutation on other androgen-sensitive variables related to Ar such as anatomical sexual dimorphisms in the brain or physiological sexual dimorphisms such as bone deposition.

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Appendix B

I would like to thank Dr. Ann Dorward for the work she performed during the Androgen Sensitivity Test. She performed the castration surgeries on all of the mice as well as the capsule (empty or T) implementation surgeries.