EVALUATION OF THE INVOLVMENT OF THE ANDROGEN RECEPTOR IN MOTIVATED BEHAVIOUR USING MALE RATS CARRYING THE TESTICULAR FEMINIZATION MUTATION

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ABSTRACT

Androgen insensitive male rats carrying the Testicular Feminization mutation (TFM), a genetic mutation in the androgen receptor, have observed to display sexual performance abnormalities. The nature of this deficit has not been fully described, but androgens and estrogens are important in mediating sexual behaviour in normal (wild type; WT) males. This dissertation examined pre-copulatory behaviours (appetitive) and re-examined sexual performance (consumatory), as well as anxiety levels, in order to determine the contribution of this mutation to these behaviours. A re-examination of sexual behaviour in the TFMs confirmed previous studies showing that these mutants display decreased intromissions and few ejaculations; regions within the brain important for mating displayed male like activation patterns. The accessory olfactory bulbs, important for pheromone mediated sexual behaviour, revealed the TFMs did not display morphological abnormalities, but the activation of this region was different than the WT males. An analysis of partner preference and the latency to achieve mounts did not reveal any abnormalities in sexual motivation. However, an examination of 50 kHz ultrasonic vocalizations (USVs), a precopulatory behaviour important for enhancement of feminine receptivity, revealed the TFMs displayed far fewer USVs than WT males. The expression of Foxp2, a protein linked to USV production in mice, did not differ between TFMs and WT males, however, a sex difference was found in that both groups contained higher optical densities (suggesting higher protein content) than females. Estrogen receptor alpha levels were feminized in certain regions of the TFM brain important for sexual behaviour. In terms of anxiety levels, TFMs displayed masculine levels, but displayed abnormal motor activity. Overall, the data support the conclusion that some appetitive and consumatory sexual behaviours are affected by the androgen receptor (TFM) mutation, and these abnormalities may directly or indirectly contribute to the inability of the TFMs to display sexual behaviour.

Keywords: Testicular Feminization Mutation, Androgen Receptor, Male Sexual Behaviour, Rat, Ultrasonic Vocalization, Foxp2, Estrogen Receptor,

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1.1   Introduction:

1.1.1   Differences in Sexual Behaviour

Clear differences exist between male and female rats in regards to reproductive or

mating behaviour. Masculine sexual behaviour is divided into two generalized phases,

appetitive and consumatory (Meisel and Sachs, 1994). Behaviours which encompass the

appetitive phase include pursuit and anogenital investigations of the female, as well as the

production and emission of 50 kHz ultrasonic vocalizations (Brudzynski, 2005). If the

male determines that the female is behaviorally receptive, based on anogenital

investigation and/or her behavioural displays, then he may precede to the consumatory

phase, which is characterized by mounts, intromissions, and ejaculations (see below)

(Pfaus, Kippin, Coria-Avila, 2003). The display of lordosis by the female is necessary

for sexual behaviour to take place; this arched back posture is elicited via the male by

palpation of the female's flanks. Once lordosis is displayed, the male begins a series of

mounts, with pelvic thrusts, in an attempt to insert his penis (termed intromission).

Before a single ejaculation takes place, the male may display several mounts and

intromissions; multiple penile insertions on the male's part promote sperm transport in the

female, as well as prolonging corpora luteal functioning, allowing the uterine wall to

develop in preparation for implantation of the fertilized egg (Nelson, 2000). Following

the display of ejaculation, the male rat typically ignores the female for a short period of time (usually between 3-5 minutes) and may retreat to a corner of the testing arena where he may begin to emit a 22 kHz ultrasonic vocalization (Pfaus, Kippin, Coria-Avila, 2003). Following this period of inactivity, termed the post ejaculatory period, the male may begin to display mounts and intromissions again. The number of times the males ejaculates varies, but he usually mates to exhaustion and does not return to proper form for a couple of days (Beach and Jordan, 1956). The execution of these consumatory aspects of mating could not take place without cooperation from the female. Every four to five days, the female becomes sexually receptive and this coincides with increases in serum estrogen (17beta estradiol) and progesterone (Beach, 1976). Indeed, the circuitry mediating the display of lordosis is dependent upon estrogen, which acts upon certain neuronal populations to 'prepare' the female for sexual receptivity (reviewed in Lee, Devidze, Pfaff, Zhou, 2006). Ovariectomy (which removes the major endogenous source of estrogen and progesterone) results in the inability to display lordosis, whereas hormone replacement therapy restores this reflex.

The display of masculine sexual behaviour (appetitive and consumatory behaviours) also depends upon steroids secreted from the gonads (Meisel and Sachs, 1994). Castration of males leads to an eventual cessation of copulatory activity, whereas hormone replacement therapy returns this behaviour after a given period of time (usually 21 hours/day of hormone stimulation; McGinnis, Mirth, Zebrowski, Dreifuss; 1989). However, females ovariectomized and given gonadal steroids, such as testosterone, may display mounts, but rarely show intromission and ejaculation like behaviour. Additionally, castrated males given the proper regime of estrogen and progesterone do

not readily display lordosis. In order for these animals to display either masculine or feminine sexual behaviour, hormone exposure must take place during a critical window of development (Sachs, Pollak, Krieger, Barfield, 1973). However, before an understanding of how hormones affect the development of differences in behaviour, an outline of the contribution of genes to the differentiation of the hormone secreting organs (testes and ovaries) must first be outlined.

1.1.2 Sexual Differentiation (see Figure 1)

Sex differences in the external genitalia of mammals are the end result of the difference in the sex chromosome make up of the individual (reviewed in Arnold, 1996). X chromosomes are exclusively produced by the maternal donor, whereas the paternal donor can contribute either an X or Y chromosome. Chromosomal sex determines genital sex due to the presence or absence of the *SRY* gene (sex determining region of Y). Translation of *SRY* into the protein, sry, signals the germinal ridge (i.e., the undifferentiated gonad) to develop into a testis, which begins secreting two important hormones: testosterone and Mullerian inhibitory hormone (MIH). Testosterone secretion in males directs the development of the primordial accessory sex organs to eventually differentiate into the vas deferens and seminiferous tubules, whereas MIH causes the regression of the Mullerian system, which eventually differentiates into the fallopian tubes, uterus, and cervix in females. Thus in the absence of a Y chromosome and a lack of an androgenic signal, the Mullerian system is spared (the Wolffian system degenerates), and the primordial gonads develop into ovaries. Translocation of the SRY gene onto the X chromosome, spontaneously (Vilain & McCabe 1998) or experimentally (Arnold, Xu, Grisham, Chen, Kim, Itoh, 2004), leads to a sex reversal in that XX

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individuals that contain the SRY gene develop into phenotypic males, whereas XY individuals that do not have the SRY gene develop into phenotypic females. Development of the external genitalia in males is also under the direction of androgens, however, testosterone is converted to a more potent androgen, dihydrotestosterone (DHT) (Imperato-McGinley, Binienda, Arthur, Mininberg, Vaughan, Quimby, 1985). The lack of an androgenic signal results in the development of the feminine external genitalia. Lack of masculinization of the external genitalia due to mutation or deletion of 5 alpha reductase in XY males results in a syndrome, known as Guevedoces in the Dominincan Republic, in which the individuals display ambiguous genitalia (Nelson, 2005). However, due to the rise in testosterone during puberty, the genitalia masculinize, but only to a certain extent.

1.1.3 Testosterone and Dihydrotestosterone

The Leydig cells, contained within the testes, are the major sites of production and release of androgens, such as testosterone in the male (Nelson, 2005). The testes produce androgens through the signaling of a trophic hormone, leutenizing hormone (LH), released from the anterior pituitary; subsequent production of testosterone negatively feeds back on the hypothalamus (and other structures) to shut down the release of LH; a decrease in LH eventually leads to a drop in testosterone production, which eventually leads again to the production and release of LH (de Kretser and Phillips, 1998). Thus, testosterone cycles up and down throughout the day.

Testosterone has a characteristic chemical structure (i.e., backbone), which is common to all steroid hormones, in that it is composed of three six carbon rings and one five carbon ring (Nelson, 2005). Each carbon is referred to by a number from 1 to 21.

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Cholesterol, the precursor to all vertebrate steroid hormones, is enzymatically converted into a progestin, which is subsequently converted into testosterone via cleavage of an ethyl group from carbon number 17. There are two major metabolites of Testosterone that have biological activity; dihydrotestosterone (DHT), a very potent version of testosterone, which is formed via the actions of the enzyme, 5 alpha reductase; DHT acts upon the androgen receptor in a similar manner as testosterone does in order to change the rate of synthesis of new proteins. Estrogen, the other major metabolite, is produced via the actions of the enzyme aromatase (also known as cytochrome p450 side chain cleavage enzyme) but once formed, estrogen acts upon the estrogen receptor (alpha and beta isoforms; discussed below). Thus, when examining the effects of gonadal steroids such as testosterone upon physiology and behaviour, it has to be understood that testosterone can exert its effects through the androgen and/or the estrogen receptor. Additionally, while DHT cannot be converted to estrogen, and *vice versa*, DHT can further be reduced to an active compound, known as 3β diol, which binds with high affinity to estrogen receptors and mimics the effects of estrogen in stimulating a number of different behaviors (Morali, Oropeza, Lemus, Perez-Palacios, 1994). Testosterone, upon production, is released directly into the bloodstream and is carried to various tissues via androgen binding proteins; specificity of testosterone's actions is achieved through the binding of the appropriate androgen, or estrogen, receptor.

1.1.4 The Androgen Receptor

The androgen receptor belongs to the steroid receptor superfamily of ligand activated transcription factors. The receptor superfamily contains a number of subfamilies; androgen receptors, glucocorticoid receptors, mineralocorticoid receptors,

and progesterone receptors comprise the 3C subfamily. The androgen receptor is located

on the long arm of the X chromosome, near the centromere, at position Q11-12 (Lubahn,

Joseph, Sullivan, Willard, French, and Wilson, 1988; Brown, Goss, Lubahn, Joseph,

Wilson, French, Willard, 1989). The open reading frame (about 2757 base pairs;

Quigley, Bellis, Marschke, el-Awady, Wilson, French, 1995) contains 8 exons, where the

first exon codes for the amino terminus domain (NTD), which contains the activator

functions (AF1 and AF2) important for interaction with other proteins (such as heat

shock proteins which normally bind to keep the AR inactive); exons one and two code for

the DNA binding domain (DBD), with the last five exons coding for the carboxy

terminus in which the hinge region and ligand binding domains (LBD) are located

(Gelman, 2002). The structure of the androgen receptor is highly conserved among

mammals. The homology of the DBD and LBD is very well conserved from frogs to

humans; the DBD itself is 100% identical between rats and humans. However, the NTD

is highly variable amongst species, and this is likely the reason the protein functions

distinctly in different animals (Gelman, 2002). Moreover, this region contains a high

copy number of the amino acid sequence, CAG, which codes for a glutamine. Abnormal

expansion in the number of glutamine repeats results in inherited forms of neuromuscular

disease such as Kennedy syndrome and spinal and bulbar muscular atrophy (Gelman,

2002).

1.1.5 Genomic and Non Genomic Actions of Androgen Receptor Activation

The accepted model of steroid receptor action entails the binding of the androgen

receptor, for example, by its ligands, testosterone, which induces a conformational

change in the receptor (see Figure 2). This change in shape allows the ligand bound

receptor to bind (dimerize) with other bound androgen receptors and also removes the inhibitory heat shock proteins that normally keep the protein inactive. The protein dimer is then transported to the nuclear compartment where it can interact with the chromatin at a proper site (termed the androgen response element; ARE) to initiate the transcription of androgen responsive genes and subsequent protein production. The ARE sequence is suggested to be 5'GATCAT<u>AGTACGTGATGTTCT</u>CAAGATC-3' (core recognition sequence is underlined) as determined by gel mobility shift assay and mutation analysis (De Vos, Claessens, Winderickx, Van Dijck, Celis, Peeters, 1991); however, this is far from conclusive as other ARE's have been identified. The binding of the AR complex to an ARE is important for initiating transcription via activation of an RNA polymerase, and for recruiting other proteins to amplify testosterone's effects on protein production. Several target proteins have been reported to be under regulation by androgens; some examples include calcitonin gene related peptide (Monks, Vanston, Watson, 1999), tyrosine hydroxylase (Jeong, Kim, Kwon, Kim, Seol, 2006), the androgen receptor itself (Kerr, Allore, Beck, Handa, 1995), and the two enzymes important for converting testosterone into active metabolites (DHT and estrogen), 5 alpha reductase and aromatase (Karolczak, Küppers, Beyer, 1998). The process outlined above is termed a 'classical genomic response' and can take several hours before protein production is achieved; however, while less studied, non classical responses have been reported to exist for the androgen receptor (see Figure 3). Revelli, Massobrio, and Tesarik (1998) list six features of androgens that distinguish them as non genomic in action: 1) rapid effects (from seconds to minutes), which essentially rules out the possible production of mRNA, 2) can be accomplished in cells that do not produce mRNA or cell clones that do not have

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androgen receptors, 3) can be initiated by androgens complexed to high molecular weight proteins (such as bovine serum albumin) that do not cross the cell membrane, 4) the rapid effects persist in the presence of transcriptional inhibitors, 5) cannot be blocked with traditional nuclear receptor antagonists, and 6) are steroid specific. However, some of these listed features appear to be specific for a membrane bound androgen receptor that does not share homology with the traditional androgen receptor, and seems to exclude the rapid effects that are possibly induced by the well characterized cytoplasmic androgen receptor. Androgen treatment has been shown to increase the intracellular concentration of Ca^{++} via regulation of a voltage sensitive Ca^{++} channel; Ca^{++} influx results in a cascade of responses such as the activation of protein kinases (reviewed in Revelli, Massobrio, and Tesarik, 1998), and may induce transcription of a number of proteins that do not contain a traditional ARE (reviewed in Rahman and Christian, 2007).

1.1.6 Distribution of the Androgen Receptor in the Nervous System

As stated, androgens exert genomic and non genomic effects in target tissues which contain an androgen receptor. The distribution of androgen receptor mRNA has been characterized in rats; while AR mRNA was widely distributed throughout the nervous system, the regions which contained the darkest signal (and thus, high amounts of expression) were the medial preoptic area, bed nucleus of the stria terminalis, ventromedial hypothalamus, medial amygdala, and hippocampus (Simerly, Chang, Muramatsu, Swanson, 1990). Interestingly, most of these areas have been implicated in the regulation of male and female reproductive behaviour and hormone secretion. Other areas which possessed AR mRNA included regions which process auditory information (such as the dorsal cochlear nucleus), vestibular information (such as the nucleus of the

solitary tract), and olfactory information (such as the olfactory bulbs). Additionally, AR

mRNA was found in the motoneurons of a number of different cranial nerve nuclei, as

well as ventral horn motoneurons, suggesting androgens influence a large number of

processes within the nervous system. Prominent sex differences in the protein

distribution of the androgen receptor have been reported in rats; males displayed higher

levels of AR expression in the lateral septum, the bed nucleus of the stria terminalis, the

medial preoptic nucleus, the anterior hypothalamic area, the arcuate nucleus, the

corticomedial nucleus of the amygdala, and the ventromedial nucleus when compared to

females (Roselli, 1991; McGinnis and Katz, 1996).

1.1.7 The Estrogen Receptors

Estrogen receptors, like the androgen receptor, belong to the steroid receptor

superfamily of ligand activated transcription factors. However, unlike the androgen

receptor, there appear to be two different receptor types (isoforms) which bind estrogen

(17-beta estradiol; E2). In humans, the gene encoding the estrogen receptor alpha

isoform has been mapped to the long arm of chromosome six (6q25.1), whereas the beta

isoform is located on chromosome 14 (14q22-24). However, in the mouse, the gene

encoding ER beta is located on chromosome 12, and produces an open reading of 1455

nucleotides, encoding a protein of 485 amino acids (Tremblay, Tremblay, Copeland,

Gilbert, Jenkins, Labrie, Giguere, 1997). The murine ER alpha version has been mapped

to chromosome 10 (Sluyser, Rijkers, De Goeij, Parker, Hilkens 1988), in which a protein,

599 amino acids long, is produced from an open reading frame containing 9 exons

(White, Lees, Needham, Ham, Parker, 1987). The protein is a bit shorter in rats,

approximately 485 amino acids, likely due to differences in the number of amino acids in the amino terminus of the protein.

In terms of sequence homology, the human ER beta isoform shares approximately 85% identity to rats and 88% sequence identity to mice (Enmark and Gustafsson, 1999).

Just like the androgen receptor, the estrogen receptors have a modular structure (six functional domains, labeled A through F) with an amino terminus, containing two regions that have important transactivator functions, a DNA binding domain (characterized by two zinc finger motifs), and a C terminal domain containing the ligand binding site (LBD). Estrogen receptors display classic genomic effects, in that they can upregulate or down regulate protein production by binding to response elements (termed estrogen response elements or EREs) following ligand binding. The DNA binding domain containing the two zinc fingers is approximately 97% homologous between the two different isoforms, and thus, both likely bind to similar EREs. Analysis of the ligand biding domain reveals that this region is approximately 60% homologous between the receptors, but the binding kinetics for E2 is similar between the two isoforms (Couse and Korach, 1999). The distribution of each receptor appears to be somewhat distinct with little overlap in the periphery; ER alpha mRNA is predominant in the uterus, mammary gland, testis, pituitary, liver, kidney, heart, and skeletal muscle, whereas ER beta mRNA is expressed in the ovary and prostate. Distribution of these receptors overlaps in the epididymis, thymus, adrenals, and bone (Couse and Korach, 1999). Additionally, the distribution of the different receptor subtypes overlap in some, but not all, cell populations in the brain (Shughrue, Lane, and Merchenthaler, 1997; Perez, Chen, Mufson, 2003; Shughrue, Scrimo, Merchenthaler, 1998). For example, ER alpha and

beta are both expressed in the medial preoptic area, ventromedial hypothalamus, and medial amygdala, but ER alpha is expressed solely in the Purkinje cell layer of the cerebellum. Additionally, in the preoptic area, cells expressing both receptor subtypes have been observed (Perez et al, 1998).

Mutations in the estrogen receptor have not been reported to be embryonic lethal, however, in adulthood, an increased number of genetic mutations in the estrogen receptors have been reported to be associated with breast cancer. Depending on the type, the mutation may render the protein constitutively active (i.e., active in the absence of ligand), may result in an inactivation of other estrogen receptors, may result in a decrease or complete loss of transcriptional ability by the mutated receptor, or may result in the increased degradation of the receptor (reviewed in Sluyser, 1995).

1.1.8   Sexual Differentiation of Sexual Behaviour

An understanding of the hormonal basis of sexual development has lead to an understanding of how sex differences in behaviour (such as mating activity) are produced. As already outlined, genetic sex determines gonadal sex and the pattern and type of hormonal secretion. Androgens such as testosterone and dihydrotestosterone are important for masculinization of the reproductive tissues as a lack of androgenic stimulation results in the feminine phenotype. Masculinization of the central nervous system mimics this pattern, in that androgens are important for inducing differentiation of certain parts of the nervous system, otherwise the intrinsic developmental pattern is feminine.

In terms of male copulatory behaviour in the rat, the critical period (i.e., the time of greatest sensitivity to androgens) of development appears to be just before and just after birth (the perinatal period). Exposure to testosterone during this time results in a permanent change in the secretion of gonadotropins, which is tonic in the male and cyclic in the female (reviewed in MacLusky and Naftolin, 1981). Weisz and Ward (1980) reported that levels of androgens, such as testosterone, were significantly elevated in the plasma of males compared to females on embryonic day 18; this sex difference lasted well into the postnatal period. Treatment of pregnant female guinea pigs with various doses of testosterone resulted in the suppression of lordosis and an augmentation of masculine sexual behaviour (such as mounts) in female offspring given injections of androgens in adulthood (Phoenix, Goy, Gerall, Young, 1959). This pattern of masculinization/defeminization can also be achieved in female rats during the postnatal period; Nadler (1968) reported that females implanted with crystalline testosterone directly into the brain on post natal day five, displayed mounting behaviour in adulthood, but only if injected with pharmacological doses of testosterone. However, given that testosterone can be metabolized into either DHT or estrogen, it became important to determine which metabolite, and hence which receptor substrate, was masculinizing the brain. Paup, Coniglio, Clemens (1972) reported that females injected with the non steroidal estrogen, diethylstilbesterol, on days 2-4 postnatally displayed male mounting behaviour in adulthood (when given testosterone), whereas no mounting was observed in females given injections of androsterone, an androgen which is not converted to estrogen. Additionally, Coniglio, Paup, and Clemens (1973) subsequently reported that castration of male golden hamsters on days 2-4 postnatally displayed masculine sexual behaviour in

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adulthood if injected with androgens (such as testosterone, but not DHT or androsterone), or estrogens (such as diethylstilbesterol or estradiol benzoate) immediately following castration. Males given injections of DHT or androsterone did not display mounts even when given testosterone in adulthood.

To summarize, androgens are converted to estrogens in certain cell populations, and this is the masculinizing factor which operates to 'organize' the male brain via activation of the estrogen receptor. Interestingly, that estrogen masculinizes the brain is confusing to some as this hormone is often referred to as the 'female' hormone, whereas testosterone is referred to as the 'male' hormone.

The initial study by Phoenix, Goy, Gerall and Young (1959) formed the basis for the organizational/activational hypothesis, which is a guiding principle in the study of the effects of hormones on the regulation of behaviour. Essentially, the hormones which serve to cause permanent changes upon the nervous system (or organization) during a developmental critical period are the same ones needed for the stimulation (or activation) of either male or female sexual behaviour in adulthood. That testosterone is converted into an estrogen to organize the nervous system in the masculine direction (as outlined above) for sexual behaviour became known as the 'aromatization' hypothesis (Naftolin, Ryan, Petro, 1971). Recent genetic studies, in which the estrogen receptor was 'deleted' from the genome of mice, supported the aromatase hypothesis in that males with a knockout of the ER alpha gene displayed reproductive behaviour abnormalities and infertility (Ogawa, Lubahn, Korach, Pfaff, 1997). In contrast, knockout of the ER beta gene did not appear to produce any effects on sexual behaviour and reproductive success (Krege, Hodgin, Couse, Enmark, Warner, Mahler, Sar, Korach, Gustafsson, and Smithies,

1998). However, deletion of the gene encoding aromatase resulted in deficits of

consumatory sexual behavior as male knockout mice displayed increased latencies and

decreased frequencies of mounts (Honda, Harada, Ito, Takagi, and Maeda, 1998). In

contrast, though, another study reported no obvious phenotype of males in which the

aromatase gene was knocked out (Fisher, Graves, Parlow, and Simpson, 1998). The next

section discusses how hormones shape the nervous system developmentally.

1.1.9 Sex Differences in the Nervous System and the Activational/Organizational Effects of Hormones

Several differences in the nervous system between males and females have been

reported (Breedlove, 1992). Such sexual dimorphisms may take the form of differences

in terms of volume a specific collection of cells within the nervous system (termed a

nucleus; not to be confused with 'nucleus', the membrane surrounding the genetic

material in a cell), which may be due to differences in the number and/or size of cells and

in the degree of connectivity (such as greater dendritic arborization or increased

synapses) of a given particular region. However, these are not the only types of

dimorphisms which have been reported as protein expression and neurotransmitter

release have also been observed to be different between the sexes. The study of sex

differences in the brain may give insights into how the nervous system may regulate

differences in behaviour, and the contribution of hormones to these processes. However,

it should be recognized that non hormonal mediated mechanisms that produce these

sexually differences have been suggested to be important in differentiation of the brain

and spinal. Indeed, using microarray technology, it has been observed that there are a

number of transcripts that are sexually dimorphic before differences in hormone titers

arise (for example, see Dewing, Shi, Horvath, Vilain, 2003). What follows are a few exemplars of how gonadal steroids shape the nervous system of males and females to produce sex differences in structure which ultimately mediate differences in behaviour.

1.1.9.1 Medial Preoptic Area

A very prominent sex difference has been observed in the medial preoptic area in the hypothalamus. This region is approximately five times larger in volume in males compared to female rats (Gorski, Gordon, Shryne and Southam, 1978); additionally, a region located within the central portion of the MPOA is also sexually dimorphic and is termed the sexually dimorphic nucleus of the preoptic area (SDN-POA) (Gorski, Harlan, Jacobson, Shryne, Southam, 1980). Differences in nuclear volume can arise in several different ways. First, the number of cells being created (termed neurogenesis) could be sexually dimorphic, the number of cells migrating to the area could be higher in males, the number of cells dying could be different, or cells may be differentiating into different phenotypes in one sex but not the other. In terms of the SDN-POA, there was no evidence to support the notion that there was increased neurogenesis (Dodson, Shryne, Gorski, 1988) or cell migration (Jacobson, Davis, Gorski, 1985) in males compared to females. Instead, it was noted that females displayed higher rates of programmed cell death during ontogeny of the SDN-POA compared to males (Davis, Popper, Gorski, 1996); these studies observed the number of pyknotic cells (dead cells) or the incidence of fragmented DNA (a characteristic of cell death) via the use of a TUNEL stain. This sex difference can be accounted for by the organizational actions of androgens acting during a critical developmental period in males. Neonatal castration and injection of testosterone attenuated apoptosis, whereas neonatal castration followed by injection of

the vehicle produced rates of cell death similar to gonadally intact females. The major

androgenic metabolite, estrogen, was subsequently shown to be important for attenuating

cell death in the SDN-POA of males compared to females (Dohler, Srivastava, Shryne,

Jarzab, Sipos, Gorski, 1984; Döhler, Coquelin, Davis, Hines, Shryne, Sickmöller, Jarzab,

Gorski, 1986; McCarthy, Schlenker, Pfaff, 1993).

1.1.9.2   Spinal Nucleus of the Bulbocavernosus

The spinal nucleus of the bulbocavernosus (SNB) is another region within

the rat nervous system that displays a robust sex difference and conforms nicely to the

principles of the organization/activation hypothesis, however, aromatization is not

necessary for proper development. The SNB is made up of about 200 motoneurons (MN)

in the adult male, and is located in the lumbosacral region of the spinal cord (Breedlove

and Arnold, 1980). The SNB MNs innervate striated muscles, the bulbocavernosus and

levator ani (BC/LA), located at the base of the penis. The BC/LA muscles regulate

penile reflexes such as cups and flips, which are necessary for normal male reproductive

behaviour. Both males and females display large numbers of SNB MNs at around

embryonic day 18, as well, both sexes send afferents to the BC/LA muscles (Rand and

Breedlove, 1987), however, the cells in both males and females begin to die just before

birth, which continues up to postnatal day 7 (Nordeen, Nordeen, Sengelaub, Arnold,

1985). The sex difference in cell number is established via apoptosis, which is greatest in

the females, as the SNB system is only vestigial to completely absent in adulthood. The

lack of the SNB system in females makes sense as they do not have a penis and thus these

motoneurons are not needed. However, females can be masculinized in terms of

motoneuron number if given an injection of testosterone on the day of birth; likewise,

males can be feminized if castrated at birth or given the antiandrogen, flutamide, a specific androgen receptor antagonist.

Confusingly, reports indicated that the SNB MNs do not express an androgen receptor until PN day 7 (Jordan, Breedlove, Arnold, 1991) a time at which most if not all of the cell death had already taken place and yet testosterone was the major organizing factor. Cell death in the SNB was not attenuated in females treated with estrogen, ruling out the possibility of an estrogen mediated effect on survival (Goldstein and Sengelaub, 1990). Attention was turned to the primary targets of the SNB, as the BC/LA muscles express an androgen receptor throughout ontogeny of the system (Fishman, Chism, Firestone, and Breedlove, 1990). Subsequent studies have shown that androgens act upon the target musculature to spare the SNB cells from normal cell death (Freeman, Watson, Breedlove, 1996), likely via the retrograde transport of a 'survival' signal, such as ciliary neurotrophic factor (Forger, Roberts, Wong, Breedlove, 1993). The size of the motoneurons in the SNB is also sexually dimorphic, in that males display larger somata than female rats; additionally, castration of males results in shrinkage of the cells, whereas androgen replacement therapy returns the cross sectional size of the cells to precastration values (Breedlove and Arnold, 1981; Watson, Freeman, Breedlove, 2001). This activational effect of testosterone on soma size is dependant upon a functional androgen receptor, expressed within the motoneurons themselves (Watson, Freeman, Breedlove, 2001). However, estrogen has been shown to have an activational effect upon the connectivity of SNB cells, as treatment of males with fadrozole (a potent aromatase inhibitor) resulted in decreased dendritic branching compared to vehicle treated males (Burke, Kuwajima, Sengelaub, 1999). The effects of estrogen upon dendritic

arborization appear to rely upon the target musculature, as it appears that the muscles

indirectly support the growth of SNB dendrites (Nowacek and Sengelaub, 2006).

1.1.9.3   Medial Amygdala

The medial amygdala has also been reported to be sexually dimorphic

with males displaying a larger volume (Mizukami, Nishizuka and Arai, 1983), larger

somata (Cooke and Breedlove, 1999), and a greater number of synaptic contacts

(Nishizuka and Arai, 1981) than females, however, these differences did not manifest

until post natal day 21. The medial amygdala contains a number of different subregions,

however, a recent study reported that the number of cells within the posterodorsal

division of the medial amygdala (MePD) was also sexually dimorphic, as males

contained more cells compared to females (Morris, Jordan, Breedlove, 2008).

Masculinization of females on volumetric measures can be achieved, again, via injections

of estrogen (Nishizuka and Arai, 1981;Mizukami, Nishizuka and Arai, 1983), suggesting

morphological aspects of this nucleus conform to the organizational effects of steroids.

These results also support the aromatization hypothesis, as estrogen can influence the

volume of this collection of cells. However, it was subsequently shown that sex

differences in soma size and volume of the medial amygdala could be entirely accounted

for by the circulating levels of androgens in adulthood (Cooke, Tabibnia, Breedlove,

1999). Long term castration (greater than a month) of male rats resulted in a shrinkage of

the entire volume and size of cells in the medial amygdala, whereas androgen

replacement therapy restored these values to precastration levels. The degree of plasticity

observed in this nucleus is unprecedented in the adult mammalian nervous system but is

very reminiscent of the effects of androgens upon the song controlling areas in the zebra

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finch brain. As such, this collection of cells does not conform nicely to the traditional conceptualization of the organizational/activational hypothesis. Similar results can be achieved in the seasonal breeding Siberian hamster, *Phodopus sungorus*, by artificially inducing a light schedule which mimics the breeding season. That is, when exposed to long day photoperiods (such as would be observed in the spring and summer months), the volume of the medial amygdala and the cross sectional area of the cells within this nucleus increase in size, however, these parameters are reversed when the animal is returned to short day photoperiods (which mimic the winter months) (Yellon and Goldman, 1987). In this species, the capacity for androgens to affect the morphology of the volume and size of the cells in the medial amygdala is dependant upon photoperiod length, as differences were only detected when animals were treated in long days (Cooke, Hegstrom, Breedlove, 2002). In rats, the ability of this nucleus to remain morphologically labile to this degree into adulthood has been speculated to be a vestige, initially evolving in a seasonally breeding ancestor of the laboratory rat (Johansen, Jordan, Breedlove, 2004).

1.1.9.4   Anteroventral Periventricular

The anteroventral periventricular nucleus (AVPVn) within the hypothalamus also displays a prominent sexual dimorphism, however, in this nucleus females display more cells compared to males (Bleier, Byne, Siggelkow, 1982). Additionally, there are approximately three times more tyrosine hydroxylase (TH) expressing cells, a marker of dopamine production, in the AVPVn of females compared to males (Simerly, Swanson, Gorski, 1985). The development of this sexual dimorphism appears to be dependant upon estrogen, acting through the estrogen receptor alpha isoform (ERalpha), which,

instead of sparing cells in the female, induces cell death in males. Knockout of ERalpha in mice resulted in an increase in the number of TH expressing cells compared to wild types (WT); additionally, the number of TH expressing cells was not different between WT males and animals carrying the testicular feminization mutation (discussed below), further suggesting the androgen receptor does not play a role in masculinization of this cell population (Simerly, Zee, Pendleton, Lubahn, Korach, 1997). Thus, to date, the concepts of the organizing and activating actions of androgens, following conversion to estrogen, in shaping the nervous system and behaviour are the dominating influences in the study of hormones on physiology and behaviour. Very little attention has been given to the role that androgen receptor activation plays in shaping the nervous system and behaviour. Perhaps one reason may be the lack of animal models useful for delineating the actions of androgens, such as testosterone and dihydrotestosterone, acting upon the androgen receptor. However, rats and mice containing spontaneous mutations in the gene encoding the androgen receptor were initially identified in the 1960's and 1970's, but have not been studied extensively for several years. In recent years, mice containing a molecular switch for turning of the expression of the androgen receptor (using the cre-lox p system to flank the AR gene; Yeh et al., 2002) have been developed, and it seems that the study of the effects of androgens is experiencing a resurgence. What follows is a brief history of the androgen receptor mutation, causing androgen insensitivity syndrome, and the effects of this mutation on the brain and behaviour.

1.1.10 Androgen Insensitivity Syndrome or Testicular Feminization Mutation Syndrome

The first complete description of Androgen Insensitivity Syndrome (AIS) was made in humans that presented with internal testes but feminine external genitalia (i.e., breasts and a vagina) (Morris, 1953; although Bardin and Bullock [1974] suggest the first description was made in 1815). These individuals were karyotyped as being male (i.e., contained an XY sex chromosome complement), but displayed large breasts, a lack of pubic/axillary hair, a 'blind ending' vagina, rudimentary uterus, and internal testes which sometimes resided in the abdomen or partially descended in the underdeveloped inguinal canal. One endocrinologist, Lawson Wilkins, dubbed these patients "hairless women with testes" (in Quigley, De Bellis, Marschke, el-Awady, Wilson, French, 1995). Attempts were made to virilize these individuals via injections of various androgens, as it was thought there was a decrease or lack of androgen secretion, however, these treatments were unsuccessful (in Quigley et al., 1995). Additionally, it was subsequently noted that the abdominal testes were androgen secretory, despite being underdeveloped; given these observations, it was hypothesized that the amount of androgens released by the testes in these individuals was not enough to fully masculinize the external genitalia. While evidence to some suggested that the syndrome was due to an insensitivity to androgens in the target organs (in Quigley et al., 1995), it was also proposed that the testes secreted an estrogen like substance that was responsible for feminization of the external genitalia (i.e., it was an induced process of feminization); as such Morris (1953) coined the term testicular feminization to refer to these individuals. While it was recognized eventually that individuals with AIS did indeed suffer from insensitivity to androgens, and that "testicular feminization" was somewhat of a misnomer, the term is

still used to refer to animals (such as rats and mice) that display this androgen insensitivity syndrome.

Lyon and Hawkes (1970) identified and reported on a mouse that displayed internal testes and a feminine external genital phenotype similar to the human AIS condition; additionally, the inheritance of the mutation displayed an X linked pattern. Further studies in the 1970's on the TFM mouse showed these mutants did not display a response to androgen therapy, as well displayed a reduced binding of radiolabelled dihydrotestosterone in kidney cytosol extracts. Given the pattern of inheritance and the lack of response to androgens, it was suggested that the problem in these mice was located somewhere on the X chromosome (the location of the androgen receptor was not known at this time).

The first descriptions of the TFM rat by Allison, Chan, Stanley, Gumbreck (1971) were also similar to the human and mouse phenotypes. However, studies on the TFM rat demonstrated that pharmacological doses of testosterone could induce growth of the preputial glands (Bardin, Bullock, Sherins, Mowszowicz, Blackburn, 1973) and reduce the high endogenous amounts of leutenizing hormone and follicle stimulating hormone (Naess, Haug, Attramadal, Aakvaag, Hansson, French, 1976), suggesting some residual activity (however, it is more likely secondary binding occurred). Additionally, the internal testes of the TFM rat were shown to release high amounts of testosterone (Naess et al., 1976; Chung and Allison, 1979; Roselli, Salisbury, Resko, 1987). Nuclear uptake of radiolabelled androgens, such as DHT, in areas of the central nervous system (e.g., the amygdala, preoptic area, hypothalamus, and pituitary) and skeletal musculature of TFM rats was reported to be completely absent (Bardin and Bullock, 1974; Krey, Lieberburg,

MacLusky, Davis, Robbins, 1982; Max, 1981). Suggestions were made that the lack of

retention of DHT in the cytoplasmic compartment of cells was due to a decrease in the

amount of available androgen receptors (Gehring, Tomkins, Ohno, 1971; Bullock and

Bardin, 1973); subsequent studies reported the number of available receptors was indeed

reduced approximately 85-90% compared to normal males (Fox, 1975; Naess et al, 1976;

Wieland, Fox, Savakis, 1978). However, Fox, Blank, and Politch (1983) reported that

purification of these mutant receptors in both rats and mice and subsequent analysis of

the association kinetics of androgens showed that testosterone was able to bind normally

to the mutated androgen receptor (*It should be noted that to date, it is currently unknown

if the bound mutated androgen receptor is able to migrate to the nuclear compartment,

interact with the androgen response element(s), or recruit the necessary components to

induce genetic transcription). While *in vitro* studies indicated the mutated androgen

receptor can bind DNA on cellulose columns (e.g., see Fox, Blank, Politch, 1983), a

demonstration that TFM ARs can do this *in vivo* is still lacking.

In terms of the estrogen receptor, studies indicated TFM rats and mice bound

radiolabelled estrogen (an indication that estrogen receptors are indeed present)

(Wieland, Fox, Savakis, 1978; Olsen and Whalen, 1982), however the production of

estrogen in the mouse was observed to be to be lower in the TFM than normal males

(Rosenfeld, Daley, Ohno, YoungLai, 1977), but is elevated in the TFM rat (Roselli,

Salisbury, Resko, 1987). Additionally, aromatase activity appears to be absent in the

preoptic area and several other areas of the hypothalamus, but is normal in the medial

amygdala of TFM rats (Roselli, Salisbury, Resko, 1987).

Cloning of the human androgen receptor in 1988 allowed researchers to confirm that the androgen receptor was located on the X chromosome. Use of complementary DNA probes, obtained from the human AR sequence, to look for mutations in individuals with androgen insensitivity syndrome provided the first evidence that AIS was indeed caused by a genetic mutation (Brown, Lubahn, Wilson, Joseph, French, and Migeon, 1988). Evidence for a genetic mutation in the rat (Yarbrough, Quarmby, Simental, Joseph, Sar, Lubahn, Olsen, French, Wilson, 1990) and mouse (He, Kumar, Tindall, 1991) versions of AIS followed soon afterwards. In the TFM rat, the mutation is the result of an amino acid change (termed a missense mutation), in that guanine is substituted for the normal adenine amino acid within exon E, which lies within the steroid-binding domain of the translated protein (Yarbrough et al., 1990). However in the mouse, analysis revealed a frame shift mutation, due to a base deletion, located in the amino terminus. As a result of this base substitution, an immature stop codon is coded for down stream of the mutation, and results in a truncated androgen receptor protein (He, Kumar, Tindall, 1991). It should be noted that humans with AIS can present with differing levels of androgen sensitivity from complete to only partial forms; as such, mutations in the androgen receptor are varied in humans and may involve frame shifts or missense mutations in several parts of the gene enconding the protein. Any attempts to draw comparisons between humans and rodents must first take into account the type and location of the mutation, as well as the possible outcomes of this mutation (i.e., protein instability, reduced levels, reduced binding kinetics, etc). To date, more than 200 spontaneous mutations in the human androgen receptor have been reported, but only two mutations in the TFM mouse, and one in the TFM rat have been observed.

1.1.11 Morphological and Behavioural Effects of the TFM Mutation

Morphologically, reports indicate that the androgen receptor mutation affects the volume of the suprachiasmatic nucleus (SCN), a structure which is normally sexually dimorphic between males and females and regulates circadian rhythms (Morris, Jordan, Dugger, Breedlove, 2005). However, the volume of the sexually dimorphic nucleus of the preoptic area (SDN-POA) was unaffected, whereas, the posterodorsal region of the medial amygdala (MePD) displayed an intermediate volume (Morris et al., 2005). Soma size was observed to be reduced in the SCN and SDN-POA, but was intermediate in the MePD. Interestingly, the androgen receptor mutation affected the volume of another sexually dimorphic region in the hypothalamus, the posteromedial division of the bed nucleus of the stria terminalis, however, only on the left side (Durazzo, Morris, Breedlove, Jordan, 2007).

Behaviourally, the TFMs have been reported to display differential performance in a test of spatial reference memory, termed the Morris Water Maze (Jones and Watson, 2005). In this study, the ability of the TFMs to find the hidden platform (termed 'escape latency') in the maze was longer than the WT males on some of the test days, but shorter than the WT females on all days reported. Additionally, the androgen receptor mutation affected the morphology of the hippocampus, as TFMs displayed a larger CA1 field compared to WT females.

Studies regarding the sexual abilities of the TFMs have reported that the display of mounts and intromissions is extremely variable, with ejaculation being severely affected (Stanley, Gumbreck, Allison, Easley, 1973). In a study by Beach and Buehler (1977), it was reported that one of their TFM affected males displayed intromission and

ejaculation like behaviour despite having no penis; they suggested that the nervous system was masculinized, however, due to an inability of these animals to respond to androgens, the full display of sexual behaviour was not stimulated in the majority of the mutants. While there are a few studies that have examined sexual behaviour in these animals, there are limited data regarding appetitive behaviours. Beach and Buehler (1977) reported that TFMs will pursue and investigate the estrous female, but there have been no reports regarding other behaviours such as the production of ultrasonic vocalizations or the latency to the first mount (an indirect indication of sexual arousal). Thus, it seems clear sexual performance is compromised by the androgen receptor mutation, but problems in this area may also be due to deficits associated with appetitive mechanisms.

1.2   Overview of Experiments in the Current Dissertation

This dissertation examines the effects of the androgen receptor mutation on appetitive and consumatory sexual behaviours. The first chapter is a reexamination of sexual activity in the TFM in order to confirm that our mutated animal displays similar sexual behaviour deficits as that previously reported; this is necessary as the TFM has not been studied for almost 25 years. The latency to mount is quantified as this has not been reported and may give an indication of the nature of the deficit in sexual behaviour; if the latency is significantly longer than the WT male, this may indicate that the TFMs may not be properly sexually aroused. Additionally, an analysis of the activation of areas in the nervous system important for sexual behaviour was performed using the expression of a marker of cellular activation, called Fos, in order to determine if these areas displayed any abnormalities.

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In a similar vein, an examination of partner preference was performed in order to determine if a lack of sexual behaviour was due to a lack of desire to mate; sexually active males normally prefer to spend time with a sexually receptive female if given a choice between a male and a female in estrous, or if presented with an estrous versus a non estrous female. Thus, the TFMs were given the choice between an estrous female or a male, or were given a choice between an estrous female or non estrous female in order to determine sexual motivation. An indication of a lack of motivation to engage in sexual activity could also contribute to a lack of sexual performance.

One very important pre copulatory/appetitive behaviour is the emission of 50 kHz ultrasonic vocalization; this appears to be androgen dependant and has been observed to important for enhancing sexual receptivity in the estrous female. Thus, we examined the production of 50 kHz ultrasounds in the TFM, guided by the notion that perhaps a lack of receptivity by the female (possibly due to decreased USV emissions by the TFM) results in a lack of sexual performance. The results from this chapter lead to an examination of the expression of a protein linked to the production of UVSs, termed Foxp2, in areas such as the cerebellum and caudate/putamen. The study of Foxp2 is a very hot topic, as this protein has undergone rapid evolution in humans, and some suggest that this protein (important for learning language) was instrumental in the evolution of our species.

Previous studies also indicate that TFMs display morphological abnormalities in the hypothalamus and medial amygdala, but no attention has been given to an area in the nervous system which may be important in 'stimulating' sexual behaviour, namely the accessory olfactory bulb. The accessory olfactory bulb has been reported to be sexually dimorphic, being larger in males. Additionally, because sexual behaviour in rats and

mice appears to rely upon the detection of sex specific pheromones, and that the olfactory

bulbs process this type of information, the AOB was analyzed in the TFMs to determine

if there were any morphological abnormalities that could possibly explain the lack of

sexual performance. As such, the constituent components of the AOB, the glomerular,

mitral, and granule layers, were analyzed in terms of volume, cell size, and activation

(determined by Fos expression) in the TFMs and compared to wild type males and

females. Again, a lack of sexual performance may be due to abnormalities in the AOB of

TFMs.

Because the effects of androgens can sometimes be mediated through conversion

of testosterone to estrogen, it was important to determine the status of the estrogen

receptor in the hypothalamus and regions important for the display of sexual behaviour.

While previous studies indicated that estrogen binds in a masculine manner in the

hypothalamus of TFMs, these studies only provided indirect evidence of estrogen

receptor levels. Additionally, these studies were performed before the discovery that the

nervous system expresses different kinds of estrogen receptors, and thus it was necessary

to determine if the TFMs displayed abnormal levels of the estrogen receptor isoform

important for sexual behaviour, namely ER alpha.

Finally, because gonadal steroids can also regulate non reproductive behaviours,

such as the expression of stress related behaviours, mutations in the androgen receptor

gene could possibly indirectly affect the display of proper sexual behaviour. Anxiety

levels have received little attention in the TFMs, but studies indicated very early on that

the adrenal glands are enlarged (i.e., the zona reticularis of the medulla; Stanley,

Gumbreck, Allison, Easley, 1973) and that corticosterone levels are significantly elevated

(Stanley, Gumbreck, Allison, Easley, 1973; and unpublished observations of Zuloaga and Breedlove, 2006), suggesting these mutants may have increased levels of stress.

A flow chart of the experiments presented in the current dissertation is provided in Figure 4.[1]

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[1]. Because this dissertation is 'journal article' style, unavoidably, there is considerable overlap in information from chapter to chapter.

Figure 1 Sexual differentiation of WT males, Females, and TFM affected males.

Genetic Sex

XY $X^{tfm}Y$ XX

SRY   No SRY

Gonadal Sex

Bipotential gonad   Ovaries differentiate from
differentiates into   bipotential gonad
testes

Phenotypic Sex

Wild Type Male   TFM   Female

Testosterone   Anti-Mullerian Hormone   androgen   No Anti-Mullerian Hormone,
                                            insensitivity   No restosterone

Wolfian sytem develops: Mullerian system regresses   Wolfian System Regresses   Wolfian system regresses; Mullerian
epididymis, vas deferens,   system develops: fallopian tubes,
seminal vesicles, bulbo-   uterus, inner vagina
urethral gland, prostate

testosterone converted
to dihydrotestosterone
via 5α–reductase

scrotum and penis formed   feminine external genitalia   labia, outer vagina, and
                                                               clitoris formed

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Figure 2 Classic Action of Androgen Receptor Activation

A. Genomic Response

extracellular

Testosterone

intracellular

+

androgen receptor

cytoplasm

nucleus

DNA

ARE   transcription

protein

mRNA translation
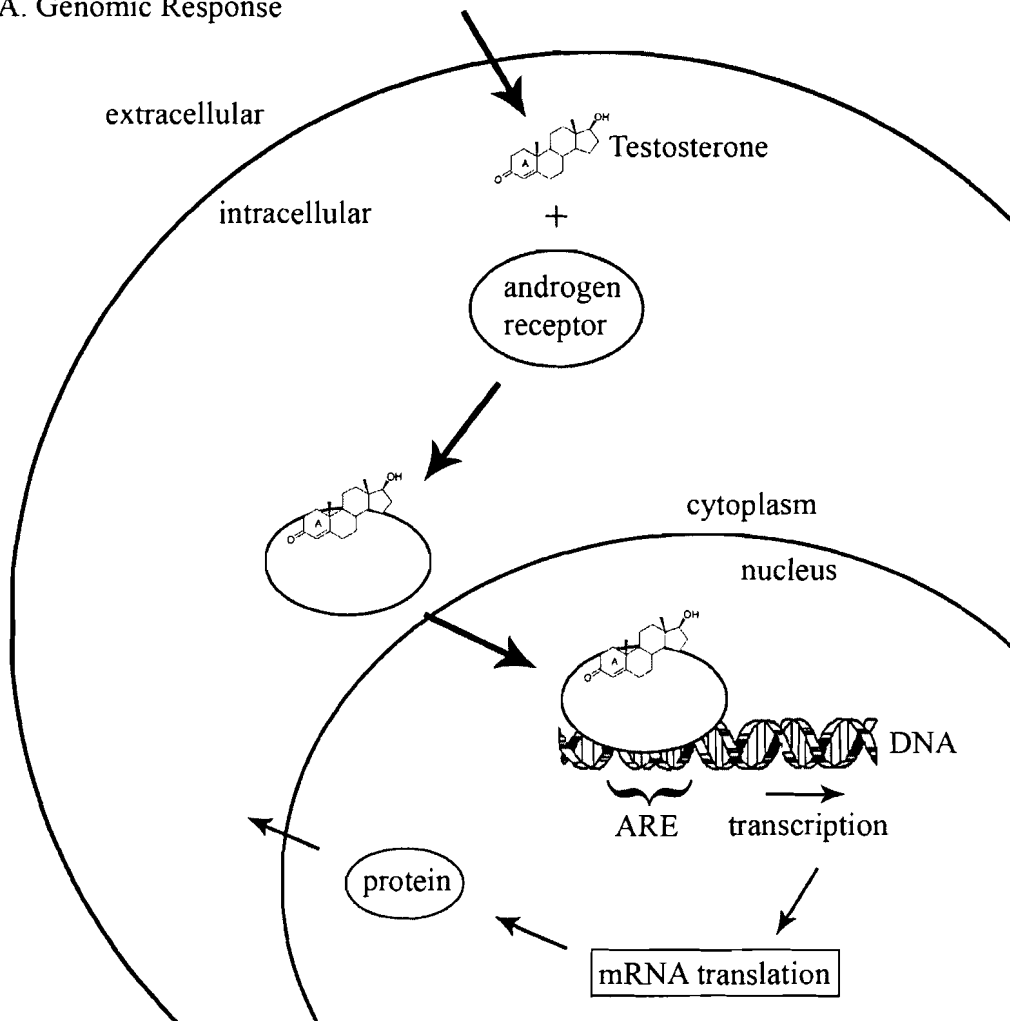
Figure 3 Rapid Non Genomic Effects of Androgen Receptor Activation

B. Non Genomic Responses
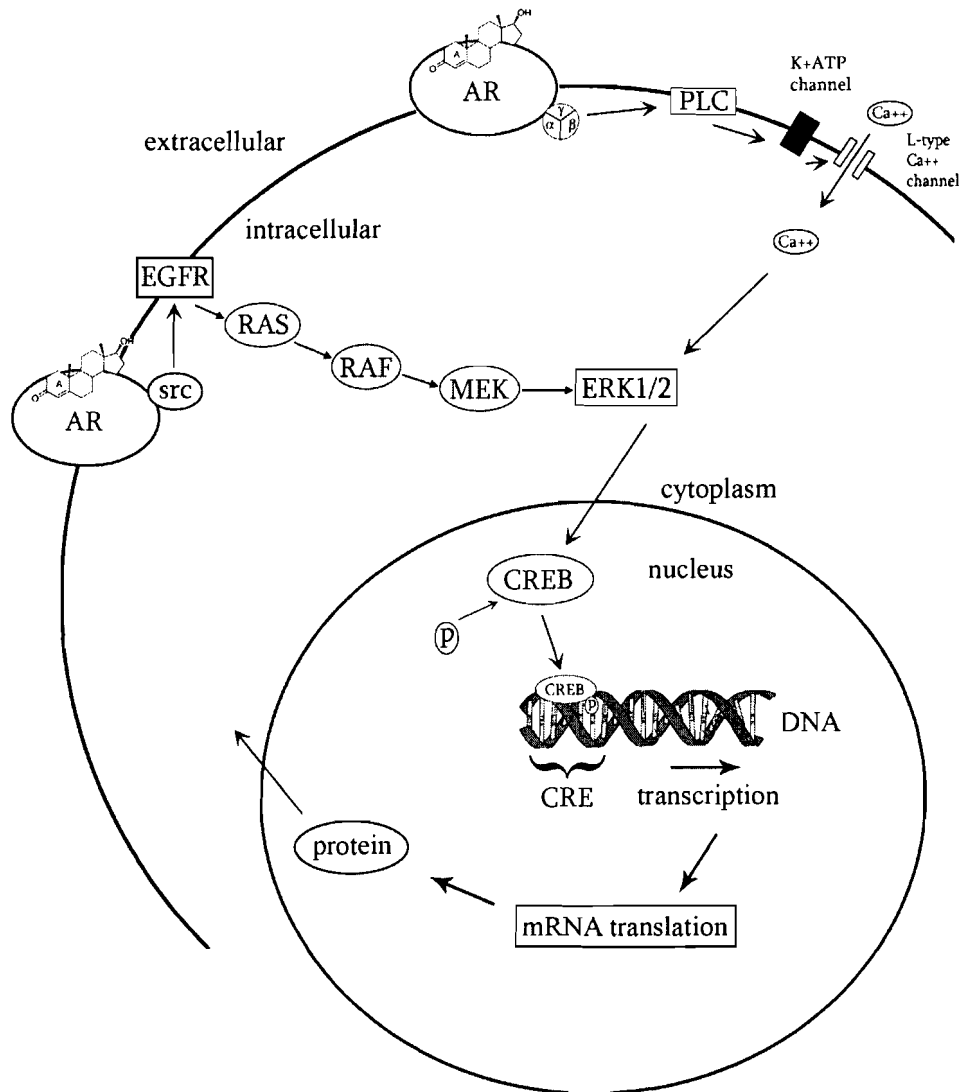
Figure 4 Flow Chart of the Experiments Presented in this Dissertation. Note: "A." represents previously reported observations in the androgen insensitive male rat (i.e., TFM rat); "B" represents the experiments in the current dissertation that were designed based upon this previous information in A.
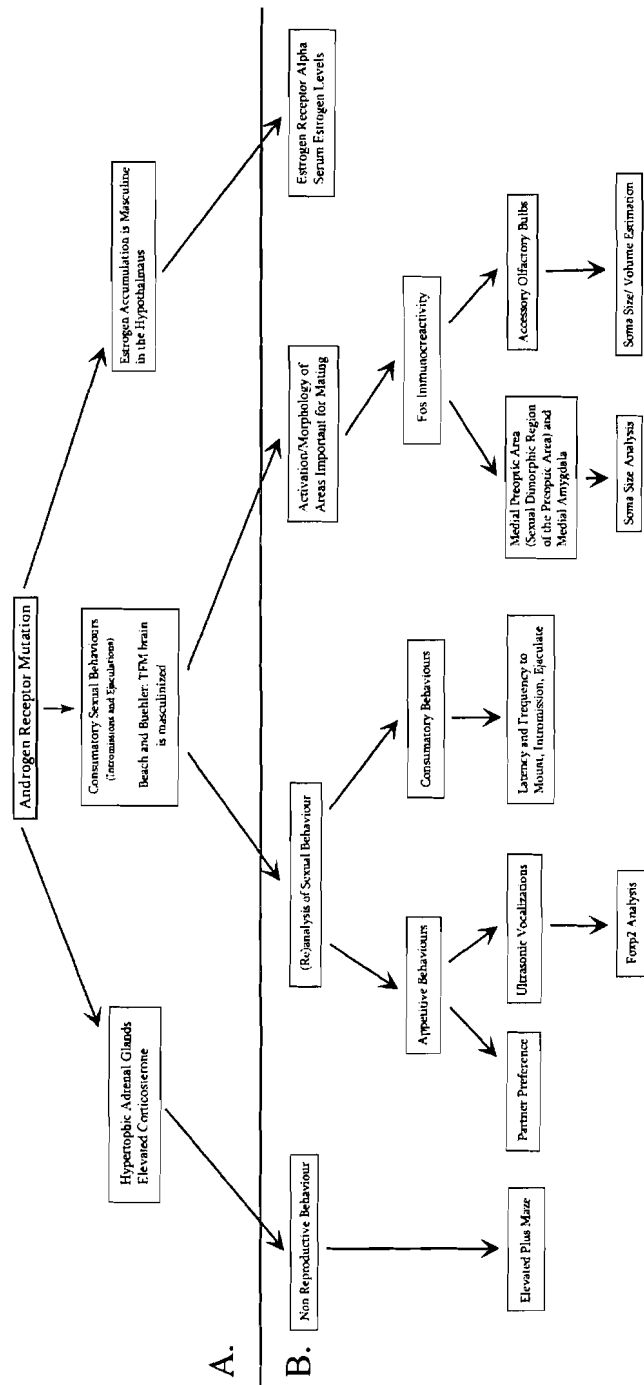
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CHAPTER 2 AN ANALYSIS OF SEXUAL BEHAVIOUR, FOS IMMUNOREACTIVITY, AND SOMA SIZE IN ANDROGEN INSENSITIVE MALE RATS

2.1 Abstract:

Male rats carrying the testicular feminization mutation (TFM) display sexual performance deficits. The mutation is in the gene encoding the androgen receptor and results in insensitivity to the masculinizing actions of androgens, such as testosterone. The virilization of the external genitalia requires androgen receptor activation during the perinatal period, thus TFMs display a feminine external genital phenotype. However, the degree to which the mutation affects the masculinization of the nervous system is incompletely understood. In order to explore this issue, we reanalyzed sexual behaviour in TFMs, quantifying several parameters of this behaviour in a standard mating arena and a bi level chamber. Additionally, we analyzed the expression of Fos immunoreactivity (a marker of cellular activation; Fos-ir) in the medial preoptic area (MPOA) and medial amygdala (MeA), two areas important for sexual behaviour, of wild type (WT) males and TFMs. Soma size was also quantified in these regions, as well as the facial nerve nucleus, of TFMs and compared to both WT males and females. As previously reported TFMs displayed sexual performance deficits (decreased intromissions and ejaculations), however, mount latencies (an indication of sexual arousal) were male-like in the TFMs. Fos-ir was comparable between WT males and TFMs in the MPOA, however, TFMs displayed more Fos-ir in the medial amygdala. The somata in WT males were larger than the TFMs and WT females, whereas no differences were detected between the groups in

the medial amygdala. However, both WT and TFM affected males displayed larger somata in the facial nerve nucleus compared to the WT females. The data suggest that, despite the smaller somata in the medial preoptic area, the amygdala and medial preoptic area become activated normally in the TFMs. Additionally, the normal mount latencies displayed by the TFMs suggests these animals do not display abnormalities in arousal. Instead, the mutation seems to affect specifically aspects of sexual performance, such as the display of ejaculations.

2.2 Introduction

Inherited androgen insensitivity syndrome (also known as testicular feminization mutation syndrome or TFM syndrome) has been reported in a number of species such as humans (Wilson, Harrod, Goldstein, Hemsell, MacDonald, 1974), rats (Stanley, Gumbreck, Allison, Easley, 1973), and mice (Lyon and Hawkes, 1970). In TFM rats, a point mutation (A to G at position 2201) in the steroid binding domain of the androgen receptor (AR) gene codes for a defective protein (Yarbrough, Quarmby, Simental, Joseph, Sar, Lubahn, Olsen, French, Wilson, 1990). This amino acid substitution potentially affects nuclear translocation and chromatin interaction capabilities (Black and Paschal, 2004). Additionally, studies have indicated that while binding of androgens to the mutated AR is normal, total binding capacity is severely reduced likely due to a decreased reserve of ARs in the nervous system and periphery (Naess, Haug, Attramadal, Aakvaag, Hansson, and French, 1976). Because the gene for the androgen receptor is located on the X-chromosome, males inheriting the mutation (TFMs) are severely affected. Phenotypically, TFMs display a feminine exterior, such as a vagina, a nipple line, and a short anogenital distance, however, they contain internal testes that are

androgen secretory (Purvis, Haug, Clausen Naess, and Hansson, 1977; Roselli, Salisbury, Resko, 1987).

The few studies regarding sexual behaviour in the TFM-affected rat are mostly incomplete and do not define the exact nature of the deficit in copulatory abilities. Shapiro, Goldman, Steinbeck, Neumann (1975) reported one of the first studies examining sexual behaviour in the TFMs. In this study, TFMs, WT males, and females were gonadectomized and injected with various hormone regimes to activate gender specific sexual behaviour. WT males and females displayed appropriate gender specific sexual activity, however, the TFMs displayed neither masculine nor feminine sexual behaviour when primed with either androgens or estrogens and progestins. This suggested to the authors that these animals were not organized in either direction on measures of sexual behaviour. It may have been the case, however, that the hormonal regime used was inappropriate to stimulate sexual behaviour. Additionally, the mating tests lasted for only five minutes in which an animal was deemed to be masculinized for copulatory behaviour if two mounts with pelvic thrusts were displayed. Thus, it appears that the mating tests were not long enough to gage copulatory ability accurately, and the criterion for establishing masculine sexual behaviour was too rigorous as it excluded mounts without pelvic thrusts. In this regard, the abilities of the TFMs were not described in enough detail to give a complete picture of the nature of their deficit.

In a follow up study, Shapiro, Levine, and Adler (1980) again tested the TFMs on measures of sexual behaviour. This time, sexual behaviour was observed in approximately 60% of gonadally intact TFMs compared to WT males and females. Hormone replacement therapy was again used, however, both females and TFMs were

given electric shock prior to behavioural testing, and the females used were induced to be hyper receptive and aroused by vaginocervical stimulation before placement in the mating arena. Moreover, the TP doses used were pharmacological, which would have resulted in higher than normal serum TP levels (Smith et al, 1977); thus, non specific binding may have occurred. Again, it is difficult to understand the full picture of the TFMs' sexual abilities from this study.

Olsen (1979) observed that in TFM affected males estrogen was able to stimulate sexual behaviour as effectively as testosterone, but DHT was ineffective. This suggests that a failure in the conversion of testosterone to estrogen *in vivo*, via aromatase, may be the basis for the lack of sexual behaviour in the TFMs. However, Roselli, Salisbury, and Resko (1987) have reported that while aromatase activity is reduced in the TFMs in several hypothalamic areas, it functions normally in the amygdala; additionally, serum estrogen levels are reported to be higher in the TFMs compared to WT males (Roselli, Salisbury, Resko, 1987). Finally, TFMs bind radiolabelled estrogen in a similar manner compared to WT males in the hypothalamus, suggesting estrogen receptor levels are unaffected by the mutation (Olsen and Whalen, 1982). Therefore, although we do not know for certain about intraneuronal estrogenic function in TFMs, the bulk of the evidence suggests some other problem may account for the sexual dysfunction in TFM affected males.

Based on the appearance of the protein, Fos, several regions of the nervous system in the normal male rat have been implicated in sexual behaviour. Baum and Everitt (1992) have observed Fos immunoreactivity in the medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST), medial amygdala (MeA), and nucleus

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accumbens (NAcc) in male rats allowed to mate to ejaculation. These areas are linked to regions in the brainstem and spinal cord that have also been shown to be important for the display of sexual behaviour (Breedlove and Arnold, 1980; Hamson and Watson, 2004). Given the importance of gonadal steroids in mediating copulation, these regions are also targets for hormones as most contain both androgen and estrogen receptors, forming a steroid sensitive neural circuit (Simerly et al., 1990; Hamson and Watson, 2004). Following copulation, Fos immunoreactivity has been observed to be colocalized with androgen and estrogen receptors in the hypothalamus and amygdala in male rats (Gréco, Edwards, Michael, Clancy, 1998). To date, though, regions in the nervous system that have been reported to play a role in masculine sexual behaviour have not been fully explored in the TFM affected males. Thus, we hypothesized that a factor contributing to the TFMs inability to display the full copulatory pattern may be due to abnormal activation of these areas. Given that androgens have well known effects upon the morphology and size of cells that contain a competent androgen receptor (for example, Watson, Freeman, Breedlove, 2001), the cells in the sex circuit may also be abnormal in the TFMs, and thus we also examined soma size in Fos expressing cells. Finally, previous reports regarding TFM sexual behaviour are not fully complete, as mount latencies, which may be a reflection of a rat's sexual arousal (Pfaus, Kippin, Coria-Avila, 2003) have never been reported. Increased latencies to mount may suggest a problem with the initiation of sexual behaviour in the TFMs, which would obviously lead to sexual performance deficits. In order to gain a better understanding of the deficit displayed by the TFMs, we reassessed sexual behaviour over a number of sexual bouts

with an estrous female using two different mating paradigms (a standard mating arena and a bi-level chamber).

2.3   Methods

2.3.1   Breeding

Females (n=4) from a colony at Simon Fraser University previously identified as carrying the testicular feminization mutation were mated with Sprague Dawley stud males obtained from the University of British Columbia. Male offspring were easily identified and housed with their male littermates, however, because TFM affected males have a feminine external genital phenotype, a polymerase chain reaction and a restriction enzyme (PCR/RFLP) based assay was used to identify these animals.

2.3.2   Polymerase Chain Reaction and Restriction Analysis

This PCR/RFLP assay was adopted from Fernandez et al. (2003) with minor modifications. Briefly, the unidentified females and TFMs were anesthetized with isoflurane and ear punch tissue was obtained. Each tissue punch was mixed with 250ul of Chelex 200 (5% w/v) diluted in TRIS-Borate-EDTA (TBE; Sigma), 25ul of proteinase K (20ug/ul; Sigma), and 25ul of RNAse A (10ug/ul; Sigma) per reaction tube. Samples were heated to 55°C for thirty minutes in a water bath (vortexed at 15 minutes). Subsequently, the tubes were placed in a heating block for 8 minutes at 100°C and then centrifuged at 12000 rpm for 5 minutes. One microliter of template DNA was added to a solution containing dH2O, 10x buffer stock, dNTP (10mM each), MgCl2 (1.2nM final concentration), Taq polymerase, and androgen receptor primers (5'-GCAACTTGCATGTGGATGA-3' and 5'-TGAAAACCAGGTCAGGTGC-3'). Samples

were then placed in a thermocycler (MJ Mini) to amplify the DNA. Cycles and times were as follows: denaturation at 95°C for 40 cycles (50 seconds each), annealing at 57°C (40 cycles, 50 seconds each), and elongation at 70°C (40 cycles, 50 seconds each). Ten microliters of the amplicon was run on 3% agarose gels containing 50μl of Ethidium Bromide at 125 volts for 40 minutes. Amplification of the AR gene produced a fragment of approximately 135bp; negative controls produced no PCR product. All samples were then treated with the Sau96I restriction endonuclease (cat # R0165S; New England Biolabs, Ipswich, MA) for digestion of the AR fragment. This enzyme recognizes the sequence, GGNCC, and cleaves between the two G's, producing a single band at approximately 80bp on 3% agarose gels. The testicular feminization mutation is a point mutation in which a guanine is substituted for an adenine in the steroid binding domain (Yarbrough et al, 1990), thus the restriction enzyme does not cleave the AR into two fragments. Instead, in heterozygous females (carriers), two prominent bands (135 bp and approximately 80bp) are present, whereas the hemizygous affected males (TFMs) display a single band at 135bp (i.e., the fragment is uncleaved), and wild type females display a single band at approximately 80bp. Following PCR analysis, animals were housed, at least two per cage, with littermates of similar genotype on a reversed 12/12 light/dark schedule with food and water available *ad libitum*. Because the process takes a minimum of two days to complete, the TFM affected males were housed with their female littermates two days longer than the WT males.

2.3.3 Stimulus Females

Female Sprague Dawley rats (n=10) were ovariectomized under isoflurane anesthesia and aseptic conditions. During the ovariectomies, females were implanted

with a single 10mm silastic capsule (1.57mm internal diameter, 3.18mm outer diameter; Dow Corning, Midland, MI) containing crystalline estradiol benzoate (Steraloids, Wilton, NH). The animals then were allowed ten days to recover. On test days, females were injected with progesterone dissolved in corn oil (0.1cc bolus containing 500ug; Steraloids, Wilton, NH) four hours before testing to induce receptivity.

2.3.4 Sexual Behaviour Testing

2.3.4.1 A) Glass Aquarium Tests

All behavioural testing took place at the animals early subjective day (at approximately 1300 hours) under red light illumination in the animal colony room. Wild type (WT) Male (n=9) and TFM (n=10) rats were placed into glass aquariums (50×25×30 cm; obtained from Hagen, Richmond, BC) for a five minute acclimatization period before each test; following this, the estrous female (chosen at random from a pool of n=10) was introduced and the animals were allowed to freely copulate for thirty minutes. Each experimental animal was tested seven times, with two tests per week and at least two days in between sessions. Frequency and latency to mounts, intromissions, and ejaculations were quantified. If an animal failed to mount, intromit, or ejaculate, a latency score of 1800 seconds was assigned; if an animal performed an intromission before a mount, the intromission latency was also assigned to the mount latency.

2.3.4.2 B) Bi-Level Chamber Tests

Mendelson and Gorzalka (1987) have previously described the use of a bi-level chamber (outside dimensions= 51 x 60.5x 15 cm) in mating tests. The chamber consists of an upper and lower level (30.5 x 15 cm; 28 cm above the floor), which are connected

on either side by a set of ramps. Using this apparatus, the female is able to escape the male's advances, which has been suggested to be ecologically valid as it may more closely mimic the natural environment in which the female typically paces the bout of mating. Thus, we placed the WT and TFM affected male rats in this chamber two days following the last of seven trials of sexual behaviour in the glass aquariums. Again, each animal was given a five minute acclimatization period prior to the introduction of a sexually receptive female. Copulatory tests lasted 25 minutes and the number of mounts, intromissions, and ejaculations were quantified, as well as the latency to each of these behaviours. Additionally, we quantified the number of level changes made by the TFMs and WT males on the last test day; this has been reported to be an index of sexual arousal in which increased level changes suggests increased arousal.

2.3.5   Tissue Preparation and Fos Immunocytochemistry

Two days following the last sexual behaviour test in the bi-level chamber, WT males and TFMs were given one last sexual behaviour test with a receptive female. Following the display of seven mounts with pelvic thrusting, WT males and TFMs were left in the testing arena by themselves for one hour. These animals, plus the estrous females used as stimulus animals in this test, were killed using a mixture of O_2 and CO_2 and perfused transcardially with approximately 60cc of phosphate buffered saline (PBS; 0.1M) followed by 60cc of 4% (w/v) paraformaldehyde diluted in PBS. Brains were harvested and placed into 4% paraformaldehyde/PBS for 2 hours and then placed into a 20% sucrose (w/v) dissolved in PBS overnight for cryoprotection. Brains were blocked in the coronal plane, mounted on a frozen stage with M1 embedding medium (Lipshaw) and sliced in the coronal plane at 50μm intervals using a sledge microtome (American

Optical Company, model 860). Sections were divided into three parallel series and then stored in an antigen sparing compound, DeOlmos solution (Watson et al, 1986), and placed into the freezer (-20° C) until processed for Fos immunoreactivity (Fos-ir). Tissue for each of the groups (i.e., TFMs, WT males, and females) used in the soma size analysis (see below) was treated in exactly the same way. That is, the tissue from the WT females was also stained for Fos-ir, but this data was not quantified.

One series of brain sections containing the medial preoptic region and medial amygdala were stained for Fos-ir using the free floating method in custom made tissue trays. All sections were washed in phosphate buffered saline (PBS; 0.1 M) containing triton X-100 (Sigma Chemicals, St. Louis, MO) for 3x5 minutes between each of the incubations listed below; all incubations were done at room temperature unless otherwise stated. Sections were blocked using 10% normal goat serum (Vector Laboratories, Burlington, Ontario) and then the tissue was incubated in the primary antibody (sc 52G goat anti-fos polyclonal used at 1:2000 dilution; Santa Cruz Biotechnology, Santa Cruz, California) overnight at 4°C. According to the manufacturer, this antibody is directed against the amino terminus of the human Fos protein. A search of the NCBI data base using a basic local alignment search tool for amino acid homology (BLASTp) showed this epitope (i.e., the entire 138 amino acid sequence of the NH_2 terminus) shares 100% homology with the human Fos protein (accession number: AAC98315), with less homology to human FosB (40% homology; accession number: CAG46898), and rat fos related antigen-2 (44% homology; accession number: AAC59866). Thus, the antibody appears to be specific for members of the Fos family.

A goat anti-rabbit secondary antibody, used at a concentration of 1:200, was then added and the tissue was incubated for 1 hour (Vector Laboratories, Burlington, Ontario). Following this, the tissue was incubated with an avidin-biotin horseradish peroxidase complex (according to the manufacturer's instructions; Vector Laboratories, Burlington, Ontario). Diaminobenzidine was used to identify antigen bound sites with nickel chloride (0.3% v/v) added for intensification, containing 0.003% hydrogen peroxide; the reaction product was dark blue/black. Omission of the primary antibody was used as a negative control and resulted in unstained sections. Sections were mounted on glass slides coated with gelatin, dehydrated in alcohols, cleared in xylenes, covered-slipped using Permount (Sigma), and analyzed using a light microscope (at 200x magnification) equipped with a camera attached to a personal computer.

2.3.6 Image Analysis

Sections containing the medial preoptic area (MPOA) and medial amygdala (MeA) in the WT males and TFMs (WT females were only used for soma size analysis, see below) were captured using a 3ccd camera (Sony), the images were imported into an image analysis program (AIS/MCID), and then converted into 8-bit grey scale images. A researcher blind to experimental conditions counted the number of Fos positive cells with the aid of the image analysis software. For this procedure, a thresholding adjustment was used to highlight cells containing immunoreactivity that was deemed to be above background (i.e., all light and dark cells clearly stained for Fos). A virtual rectangle (235μm x 360μm for the MPOA; 140μm x 345μm for the MeA) was placed overtop of the areas being analyzed and the computer program automatically counted only those

nuclei that were above the threshold of detection set by the researcher; to ensure accurate counts, the results were adjusted by hand if necessary.

Following quantification of Fos-ir, slides were placed in Xylene overnight to remove cover slips. The slides were rehydrated in a series of alcohol solutions (75-100% EtOH), and Nissl substance was stained using 8% thionin. Each slide was then re-dehydrated in alcohol and re-cover slipped for soma size analysis. Twenty cells per animal, contained within the MPOA or MeA, were selected for analysis based on the appearance of Fos-ir for both the WT and TFM affected males; cells in these regions were chosen at random in the WT females (only a subset of the WT females was used; n=5). The cells were magnified to 600x, and an additional magnification of 300% was achieved using the image analysis software and this greatly improved measuring accuracy. Two dimensional areas were quantified using a tool in which the outline of the cell was traced by hand by a researcher blind to genotype of the animal; the image analysis program automatically calculated the soma size in μm^2.

For comparison, we chose to measure somata in the facial nerve nucleus (n7) as these motoneurons express androgen receptors (just as cells in the MPOA and MeA), however, these cells are not thought to be involved in copulatory behaviour. Cells were chosen at random (from two anatomically matched brainstem sections) by a researcher blind to experimental conditions; to be included in the analysis, each cell had to display a clear nucleus and nucleolus. Again, a tracing tool was used to outline the cell, and the image analysis program automatically calculated the soma size in μm^2. In total, 73 cells were analyzed from the TFMs, 48 from the WT males, and 99 from the WT females.

2.4 Statistical Analysis

Statistical analyses were carried out using SPSS (v.14.0). Copulatory performance in the standard mating arena was analyzed using multiple two-tailed T tests with alpha set at 0.05 (Olsen, 1979), except for data regarding ejaculation. These data were skewed (due to a high number of zeros) and were thus analyzed with a non parametric statistic, Mann-Whitney U (alpha=0.05). We compared the performance of the TFMs and WT males on day one (sexually naïve) with performance on day 7 (sexually experienced) using paired samples t tests with alpha set at 0.05. Sexual performance in the bi-level chamber was also analyzed using multiple 2 way t tests (alpha=0.05), however, due to the skewed distribution of the frequency of mounts and frequency and latency of ejaculations, data were analyzed using Mann-Whitney U. The TFM group was broken down based upon the litter that each animal came from (Litters 1-3; see below) and performance was analyzed using Kruskal-Wallis Test with alpha set at 0.05. Soma size was analyzed using a one way ANOVA with alpha set at 0.05; where appropriate, *post hoc* analysis was carried out using Fisher's least significant difference (LSD) statistic. To detect any possible differences in Fos immunoreactivity in the MPOA and MeA, one-tailed t-tests were employed with a significance level set at 0.05.

2.5 Results

2.5.1 Sexual Behaviour- Glass Aquarium

1) Mounting Behaviour- The average frequency and latency of mounting behaviour is displayed in figure 5. The frequency of mounting behaviour displayed by the TFMs was significantly different from the WT males on Days one (t=3.617, p=0.006), five (t=2.873, p=0.018), six (t=2.327, p=0.043), and seven (t=2.727, p=0.022) only. Interestingly, we

observed some of the TFMs to display multiple mounts with pelvic thrusting per single elicited lordosis response; we scored these exaggerated mounts as one (per lordosis), and thus the actual number of mounts is likely higher than what we are reporting. In terms of the latency to the first mount, TFMs displayed a shorter mount latency only on day one compared to the WT males ($t=5.65$, $p<0.001$); there were no other differences detected.

2) Intromission-like behaviour- The display of intromissions was quite variable among the TFMs; however, every TFM tested in the current report exhibited an intromission at least once. The only difference in the frequency of intromissions appeared on days three and seven, in which the TFMs displayed more than the WT males (day three: $t=2.461$, $p=0.035$; day seven: $t=4.026$, $p=0.001$); no other differences in intromission frequency were detected. In terms of intromission latency, the WT males and TFMs did not differ on the first four test days; differences arose on days five, six, and seven, in which the WT males exhibited lower intromission latencies compared to the TFMs (day 5: $t=4.923$, $p<0.001$; day 6: $t=7.344$, $p<0.001$; day 7: $t=6.342$, $p<0.001$).

3) Ejaculation-like behaviour- The frequency and latency of the display of ejaculation was severely affected in the TFMs; only one TFM displayed the ejaculation-like pattern (i.e., 10% of the total). WT males displayed more ejaculations and shorter latencies compared to the TFMs on all days except the first day of testing (note: WT males did not display any sexual behaviour on this day).

Sexually naïve vs sexually experienced- Repeated pairing of WT males with estrous females affected the display of sexual behaviour. By test day seven, WT males displayed more mounting ($t=4.289$, $p=0.005$), shorter latencies to mount ($t=105.899$, $p<0.001$), more intromissions ($t=9.174$, $p<0.001$), shorter intromission latencies ($t=18.723$,

p<0.001), more ejaculations (Z=2.22, p=0.026), and shorter ejaculation latencies (Z=2.2, p=0.028) compared to test day one. However, for the TFMs the analysis suggested the display of sexual behaviour remained the same with repeated pairing with the estrous female. That is, there were no differences in terms of the frequency of mounts (t=0.629, p=0.545), latency to mount (t=0.788, p=0.451), the frequency of intromissions (t=0.249, p=0.809), intromission latency (t=0.728, p=0.485), the number of ejaculations (Z=1.00, p=0.317), or the latency to ejaculate (Z=1.00, p=0.317).

Overall seven tests of sexual behaviour, the number of mounts and intromissions displayed by the TFMs was greater than the WT males (see figure 9; mounts: $T_{15}=5.4$, p=0.0003; intromissions: $T_{15}=4.122$, p=0.003).

Analysis by Litter- It appeared that some animals performed better than others in the mating arena; upon examination of the data, it appeared the TFMs with the most robust mating behaviour came from one litter, thus we decided to break the animals down by litter and compare the mating abilities of each of the groups. While TFMs from litter one displayed the most mounts over the entire seven mating tests, in terms of a day by day analysis, this number was only significantly different on test day 2 (chi square=6.2, p=0.045) (Figure 7). Additionally, the number of intromissions was significantly different on days 3 (chi square=5.85, p=0.05), day 6 (chi square=5.72, p=0.05), and day 7 (chi square=6.54, p=0.038), with TFMs from litter one displaying increased frequencies of intromissions compared to the other litters. No differences were detected between the groups on measures of latency to achieve the first mount or intromission.

2.5.2 Sexual Behaviour-Bi-Level Chamber

The latency and frequency of mounts, intromissions, and ejaculations of the TFMs and WT males in the Bi-level chamber is displayed in figure 6. Interestingly, the TFMs did not perform well in this apparatus with only 40% displaying any sexual behaviour at all in the three separate tests, whereas 100% of the WT males tested performed sexually. The results presented below, however, contain performance from both responders and non responders of the TFM genotype.

1) Mounting Behaviour- No significant differences were found in terms of the frequency of mounting behaviour between the TFMs and WT males. However, the latency to mount was shorter in the WT males compared to the TFMs on day one (t=2.261, p=0.039) and day three (t=3.864, p=0.004).

2) Intromission behaviour- No differences were detected in terms of the number of intromissions displayed by the TFMs and WT males. However, the WT males exhibited a shorter intromission latency on day one (t=3.209, p=0.008) compared to the TFMs.

3) Ejaculation behaviour- Again, the TFMs displayed little ejaculation behaviour in the bi-level chamber; however, one TFM displayed three ejaculations in the 25 minute test on day one. WT males displayed more ejaculations that the TFMs on days one (Z=3.084, p=0.002), two (Z=2.054, p=0.04), and three (Z=3.588, p=0.0003).

Subjectively, it was readily apparent that some TFMs qualitatively and quantitatively performed better than some in this test apparatus and thus we broke the TFMs down by litter and analyzed their performance (see Figure 8). Litter one consisted of n=3, litter two consisted of n=3, and litter three consisted of n=4 animals.

1) Mounting Behaviour by litter- Differences in mounting behaviour between the three litters appeared on day three only with litter one performing the majority of mounts (Chi-

square= 7.833, df 2, p=0.02). However, in terms of the mount latency, there were significant differences on day two (Chi-square= 7.6, df 2, p=0.022) and day three (Chi-square=7.6, df 2, p=0.022); in terms of ranking the latencies from shortest to the longest, the TFMs from litter one mounted faster than litter three, which were subsequently faster than litter two.

2) Intromission behaviour by litter- No differences in intromission frequency were detected on day one (Chi-square= 3.785, df 2, p= 0.151); however, the number of intromissions was significantly different on days two (Chi-square=6.485, df 2, p=0.039) and three (Chi-square=7.6, df 2, p=0.022). The latency to begin intromitting was significantly different on days two (Chi-square=7.6, df 2, p=0.022) and three (Chi-square=7.6, df 2, p=0.022). Again, the TFMs from litter one performed the vast majority of the intromissions and displayed shorter latencies compared to the other two litters.

3) Ejaculation behaviour by litter- Only one TFM from litter one displayed any ejaculations (i.e., three ejaculations on day one).

2.5.3 Fos Immunoreactivity

Three of the TFMs were removed from this analysis, as the quality of the Fos-ir was deemed poor. Fos immunoreactivity was quantified in the medial preoptic area (MPOA) and the medial amygdala (MeA) of WT males and TFMs following seven mounts (with pelvic thrusting). Figure 10 contains the histograms and representative photomicrographs of Fos-ir in these regions. The mean number per section of Fos expressing cells in the MPOA of the WT males was not different from the TFMs (t_{12}=1.124, p=0.15); however, in the MeA, Fos-ir cells were, on average, more numerous in the TFMs compared to the WT (t_{12}=2.03, p=0.033). In this final test of sexual

behaviour, the mount latency of the TFMs (65.57 +/-23.55) was shorter compared to the WT males (159.57 +/-65.45), however, this was not significant ($t_{12}=1.35$, p=0.22). Additionally, the amount of time it took for the WT males to achieve seven mounts was 645.5 +/-105.01, whereas it took the TFMs 454.25 +/-21.5; this was not statistically significant ($T_{12}=0.548$, p=0.604)(see figure 11).

2.5.4 Soma Size

We analyzed the somatic area of the Fos expressing cells in the WT males and TFMs and compared these with somata from WT females in the medial preoptic area (MPOA) and medial amygdala (MeA) (see figure 11 for histograms and photomicrographs of thionin stained facial motoneurons). Soma size in the MPOA differed by genotype ($F_{2, 20}=52.099$, p<0.001), with the WT males displaying larger somata than both the TFMs (p<0.001) and WT females (p<0.001). In the MeA, however, there was no effect of genotype on soma size ($F_{2, 20}=1.903$, p=0.175).

There was an effect of genotype on the size of cells in the facial nucleus (F= 16.926, p<0.001) in which the WT males and TFMs did not differ in soma size (p=0.151), however, both displayed larger somata than the estrous females (p<0.001 and p=0.0002, respectively).

2.6 Discussion

2.6.1 Sexual Behaviour

In the current report, in terms of the percentage of animals displaying mounts, intromissions, and ejaculations, the TFMs do not seem to differ from Shapiro, Levine, and Adler (1980). Additionally, the observation of one of the gonadally intact TFMs to

display exaggerated mounting activity as well as the ejaculation pattern has also previously been described by Beach and Buehler (1977). Interestingly, none of these previous studies have reported on the latency to achieve the first mount (ML); ML may give some indication of the ability of the mutants to become sexually aroused (Pfaus, Kippin, Coria-Avila, 2003). We observed normal mounting latencies in the TFMs, suggesting arousal was normal. Additionally, we observed that the sexually naive TFMs began mounting on day one faster than the sexually naïve WT males, which may actually suggest increased sexual arousal on the first day of testing only.

Regarding previous studies that have reported on the sexual activity of the TFMs, it appears the display of intromission and ejaculation like behaviour is completely random as some studies have observed both behaviours and some studies have not (Shapiro, Goldman, Steinbeck, Neumann, 1975; Beach and Buehler, 1977, Olsen, 1979; Shapiro, Levine, and Adler, 1980; the current report). This is an interesting observation given that these animals share the exact same mutation in the gene encoding the androgen receptor and suggests other factors may be involved. We separated our TFMs based upon litter in an attempt to shed some light on this issue. It appeared that the most robust display of copulatory activity came from animals contained within litter one. That is, these TFMs displayed decreased latencies to mount and intromit (on some but not all of the tests), as well as increased frequencies in the display of these behaviours. In fact, the animal which showed the most robust mounting behaviour (i.e., multiple mounts per single elicited lordosis) came from litter one and was the only one to display the ejaculation response in any of the tests. Additionally, the display of sexual behaviour in the bi-level chamber was quite clearly dichotomous in that, TFMs either activily pursued

and mated with the estrous female, or merely sat motionless on one of the levels and completely ignored the female. Of the animals that displayed sexual behaviour, three of the four again came from litter one. Separation of the TFMs based upon litter and subsequent analysis of sexual behaviour has not been previously reported. Because the animals were housed and treated in a similar manner following weaning, an intriguing possibility is that factors in the postnatal environment may have played a role in shaping sexual performance.

The type and quality of maternal care postnatally can influence responses to stress in later adulthood. Meaney and colleagues (reviewed in Diorio and Meaney, 2007) have observed a high degree variation in the amount of maternal licking and grooming (LG); pups which received high amounts of LG displayed a decreased stress response in adulthood compared to pups which received low LG. The quality of maternal care has been suggested to affect gene expression. In pups receiving low maternal care, the promoter region of the gene encoding the glucocorticoid receptor, which is important for responding to stress appropriately, was inhibited from transcription by DNA methylation (essentially shutting off the gene) (Weaver, Cervoni, Champagne, D'Alessio, Sharma, Seckl, Dymov, Szyf, Meaney, 2004). Normally, methylation of this region occurs during the postnatal period, however, this epigenetic modification is rarely observed in pups which receive high maternal LG, suggesting the licking actively removes the inhibitory methylation, via an as yet unidentified process (Weaver et al., 2004). Thus, it is interesting to speculate that perhaps differences in sexual performance observed in the TFMs are a result of differences in maternal care and modifications to the gene(s) important for the display of sexual activity. Anogenital licking has been observed to

increase the number of cells in the spinal nucleus of the bulbocavernosus (Moore, Dou, Juraska, 1992); these motorneurons innervate perinial musculature, which regulate penile reflexes in male rats (Breedlove and Arnold, 1980). Additionally, decreased anogenital licking affected the display of sexual behaviour; males displayed longer latencies to achieve an ejaculation and increased the post ejaculatory interval (Moore, 1984).

It may also be the case that the lack of a penis and sensory feedback could play a role in the lack of proper sexual functioning, but this does not explain why some of the TFMs display intromission and ejaculation like behaviour (Beach and Buehler, 1979). One possibility is that the genital patch (i.e., the patent vagina) of the TFMs is sending somatosensory information to the brain, which then sends the signal for ejaculation back down to the lumbar spinal cord (Truitt and Coolen, 2003), stimulating the ejaculation like response; however, it does not appear that this part of the perineum comes into contact with the estrous female in the lordosis posture (unpublished observations). Thus, it is still unknown as to how the TFMs actually display this response.

2.6.2 Fos Immunoreactivity

Fos, a marker of cellular activation, has been used to map regions that are important for the display of consumatory and appetitive sexual behaviours. Coolen, Peters, and Veening (1997) compared Fos-ir in animals that displayed just anogenital investigations to those that displayed anogenital investigation, plus mounts, intromissions, and ejaculations. Males that investigated the anogenital region of estrous females displayed Fos-ir primarily in the MeA and a related region, the bed nucleus of the stria terminalis. Following the display of consumatory sexual behaviour, regions in the MPOA, posterior amygdala, and central tegmental fields preferentially displayed Fos-

ir. Additionally, the MeA (posterodorsal division) displayed augmented levels of Fos-ir following mating to ejaculation. Thus, it appears that the MeA becomes activated following appetitive behaviours such as anogenital investigation, but this activation can be augmented following mating to ejaculation. In the current report, we killed WT males and TFMs following the display of seven mounts with pelvic thrusting and stained for Fos. Importantly, the time it took each of the WT males and TFMs to achieve the required level of mounts did not differ statistically; however, the latency to begin mounting was shorter in the TFMs compared to the males. A comparison of Fos-ir in the MPOA did not reveal differences between the two groups, whereas, the TFMs displayed increased Fos-ir in the MeA compared to the WT males. A previous study reported that Fos-ir was increased in the MeA and MPOA of masculinized females allowed to mount an estrous female compared to non mated controls (Oboh, Paredes, Baum, 1995), suggesting increments in Fos-ir were not solely due to genital somatosensory stimulation. The data in that previous study also suggested that males and females did not differ in the amount of Fos-ir in each of these regions, however, there was no report of the latency to first mount, and thus a direct comparison with the results from the current study cannot be made. Nevertheless, one interpretation of the data in the current report could be that the increase in Fos-ir in the MeA may be a reflection of increased sexual arousal, as indexed by the short latency to mount, in the TFMs. Qualitatively, it appeared the estrous females did not readily allow the TFMs to mount them, and thus differences in Fos-ir may be due to a lack of female cooperation.

Given that Fos-ir in the MPOA did not differ from that of the WT males suggests this region is activated in a similar manner in the TFMs. This suggests the failure to

display the full mating pattern by the TFMs may not be due to abnormal activation of the MPOA in the appetitive phase of sexual behaviour.

2.6.3 Soma Size

There are numerous reports linking androgen and androgen receptor activation to the regulation of soma size in the nervous system. For example, in motoneurons of the spinal nucleus of the bulbocavernosus, motoneuron size is directly regulated by androgens in a cell autonomous manner; only those cells that contained a competent androgen receptor returned to precastration values following testosterone replacement therapy (Watson, Freeman, Breedlove, 2001). In the medial amygdala, long term castration of male rats resulted in feminized soma sizes, and this effect was also reversed with testosterone propionate (TP) treatment (Cooke, Tabibnia, Breedlove, 1999). However, in the MeA, soma size can be regulated by estrogen receptor activation as long term castrated males given estrogen replacement exhibited masculine somata (Cooke, Breedlove, Jordan, 2003). Thus, in this region, the aromatization of testosterone to estrogen (followed by estrogen receptor binding and activation) is a second means by which soma size can be regulated.

We observed feminized somata in the MPOA of TFMs, but no differences were detected in the MeA between any of the groups examined in the current report. Aromatase activity has been reported in the MeA of the TFMs, but is reduced in the MPOA (Roselli, Salisbury, Resko, 1987), thus intracellular conversion appears to be necessary for estrogen to exert it effects upon soma size. Interestingly, motoneurons in the facial nucleus were not significantly different between the TFMs and WT males, but both displayed larger somata than the estrous females. This pattern of results appears to

be independent of the activational effects of androgens on soma size in this region. There is the possibility of an organizational effect of estrogen on soma size in this area as there appears to be transient expression of the estrogen receptor during a developmental critical period (Yokosuka and Hayashi, 1992; Hayashi, 1994). Given the sex difference in serum estradiol levels developmentally (Amateau, Alt, Stamps, McCarthy, 2004), this mechanism seems at least plausible, however, estradiol levels have never been sampled in the early postnatal period in the TFMs.

Regarding the MPOA, it is possible that the observed soma sizes in these regions were shaped by the differential amount of sexual behaviour displayed by the TFMs compared to the WT males. One previous study observed decreased somata in motoneurons of the SNB (these cells regulate penile reflexes) in males given unrestricted access to females in behavioural estrous (Breedlove, 1997). Following one month of *ad libitum* mating, copulating males had smaller cells in the SNB compared to males that were restricted from mating. In the current report, the overall number of mounts with pelvic thrusting was higher in the TFMs compared to the WT males, leading to the possibility that the increased display of these behaviours by the TFMs was enough to reduce their soma sizes in the MPOA to feminine levels. However, this remains extremely speculative, as the Breedlove (1997) study has never been replicated.

2.7 Conclusion

In support of previous studies regarding sexual activity of the TFMs, we have observed deficits in sexual performance but not in aspects of sexual arousal. The current report further refines our knowledge of the effects of the androgen receptor mutation on

physiology and behaviour as regions important for the display of sexual behaviour do not

appear to be activated abnormally despite smaller somata in the MPOA.

Figure 5 Sexual Performance in the Standard Mating Arena. The frequency and latency of mounts, intromissions, and ejaculations in the TFMs and WT males over seven test sessions. Note: * indicates statistically significant comparison (see text for details).
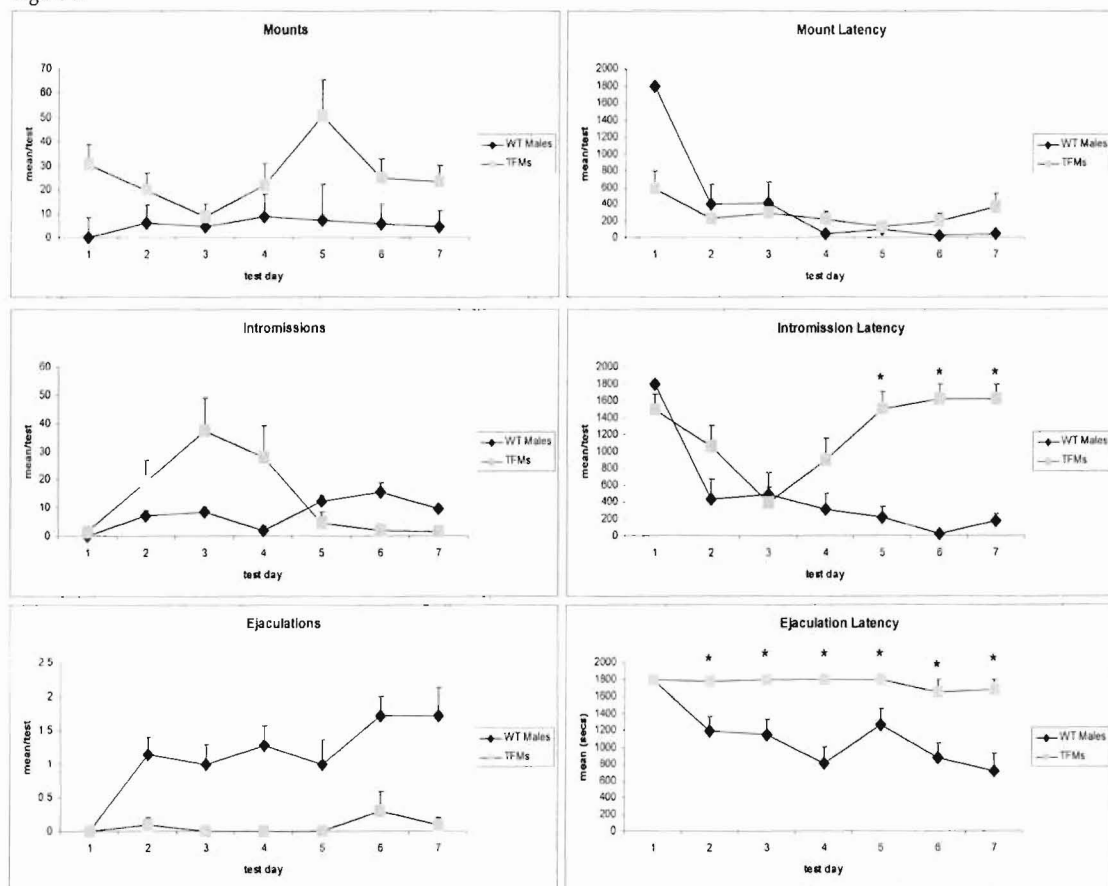
Figure 1.

Figure 6 Sexual Performance in the Bi-Level Chamber. The frequency and latency of mounts, intromissions, and ejaculations of TFMs and WT males in the Bi-level chamber. Note: * denotes statistically significant comparison (see text for details).
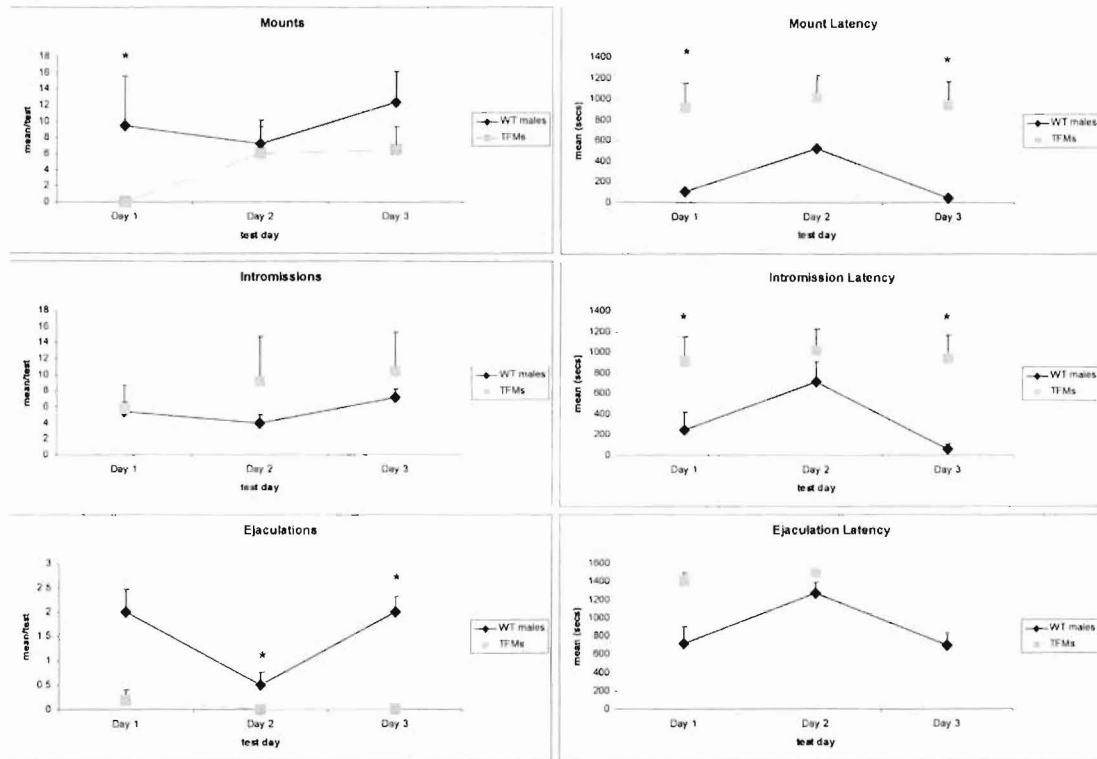
Figure 2.

Figure 7 Sexual Performance of TFMs Separated by Litter. Due to the high degree of variability, TFM sexual performance was separated by litter (cage 1, 2, or 3). Note: * denotes statistically significant comparison (see text for details).
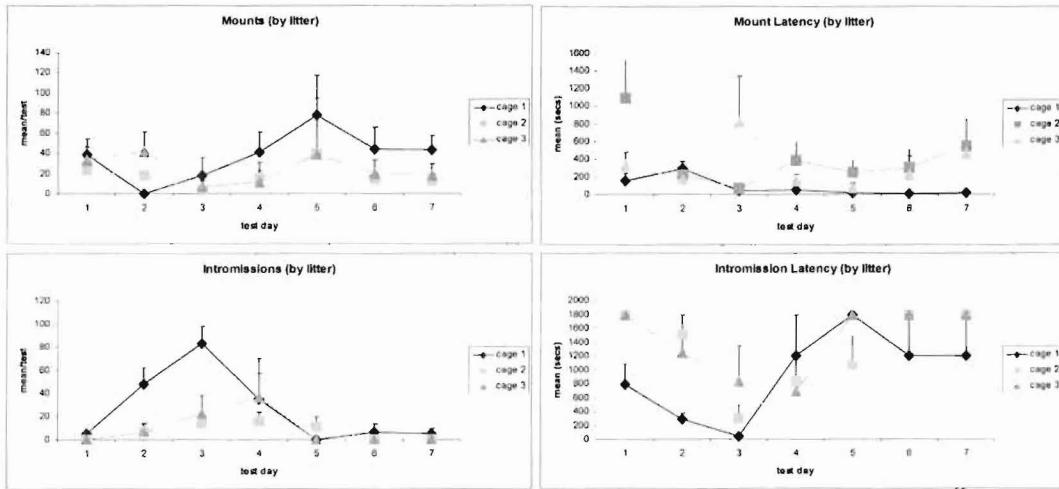
Figure 3.

Figure 8 Sexual Performance in the Bi-level chamber of TFMs Separated by Litter.Sexual activity in the bi-level chamber was clearly dichotomous as some TFMs performed sexually whereas others merely sat motionless on one of the levels. It appears that TFMs from Cage 1 displayed more robust mating activity than TFMs from the other two litters (i.e., cage 2, and 3). Note: * denotes statistically significant comparison (see text for details).

Figure 4.
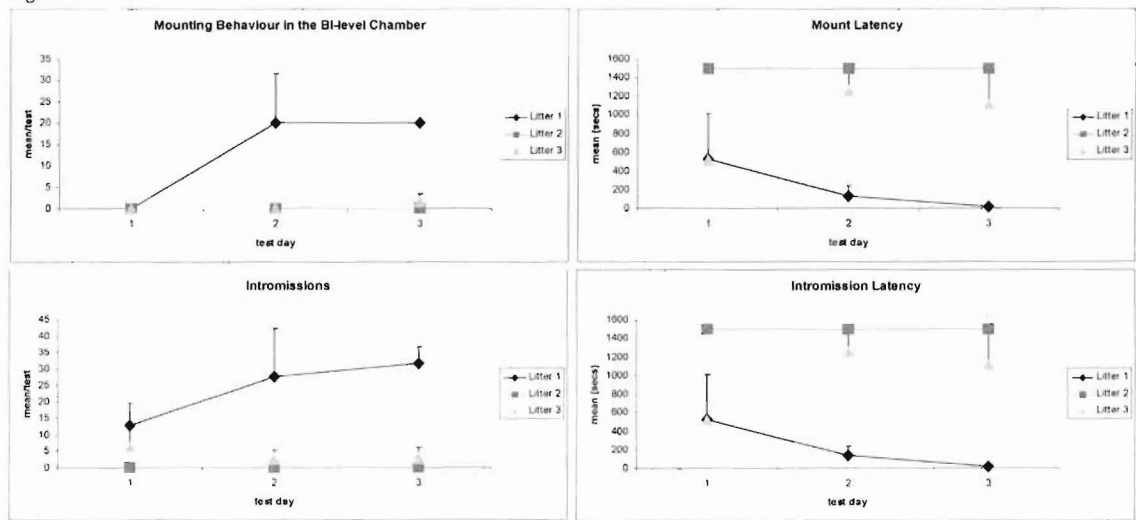
Figure 9 Overall Quantification of Mounts and Intromissions. On average, the number of mounts and intromissons per TFMs was higher for the seven test sessions compared to the WT males.
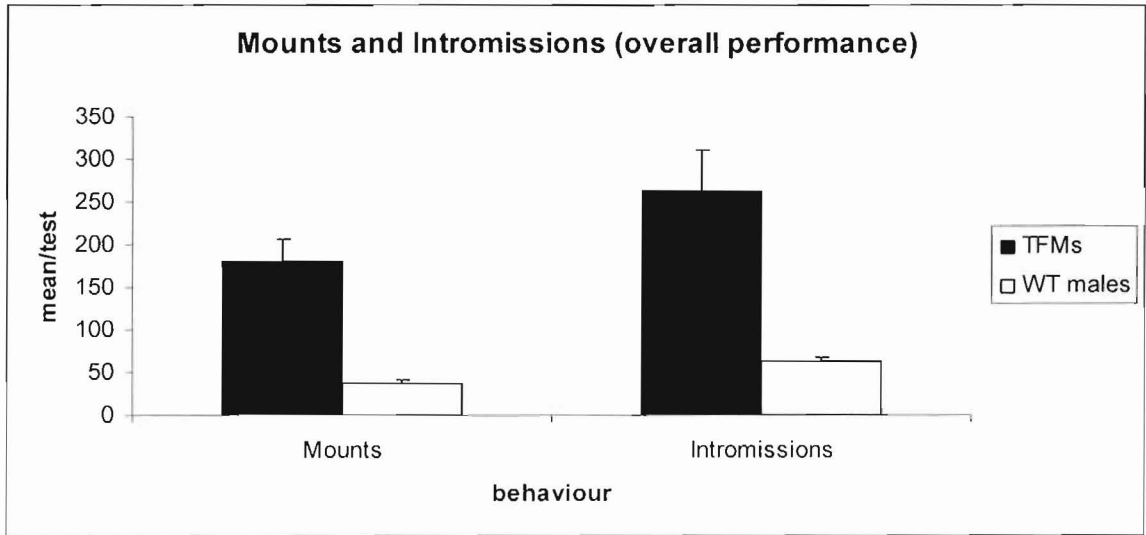
Figure 10 Fos immunoreactivity (Fos-ir) in the medial preoptic area and medial amygdala of WT
males and TFMs. TFMs and WT males displayed similar amounts of Fos-ir in the
medial preoptic area (top row), however, the TFMs displayed more Fos-ir, on average,
in the medial amygdala (bottom row). Note: * denotes significantly different than WT
male.
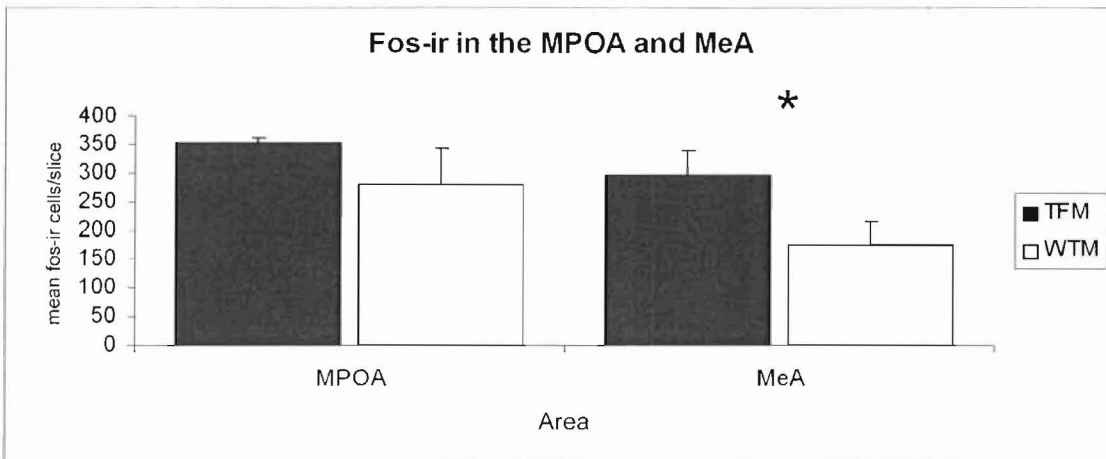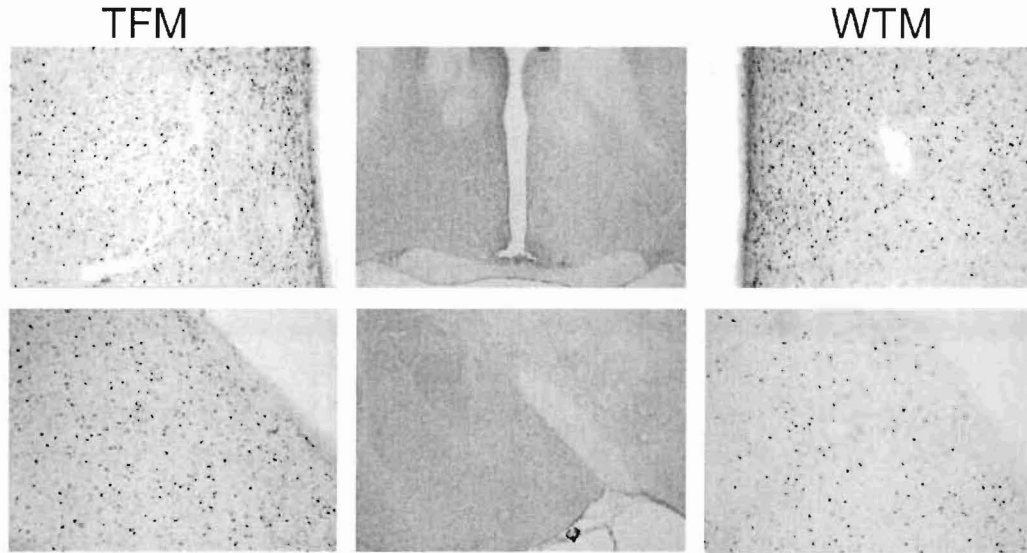
Figure 11 Soma Size in the Medial Preoptic Area and Medial Amygdala of WT males (WTM), TFMs, and WT females (WTF). The WT males displayed larger somata in the medial preoptic area compared to the TFMs (denoted by 'a') and estrous females (denoted by 'b'). No differences were detected in the medial amygdala. However, the WT males and TFMs displayed larger somata in the facial nucleus when compared to the estrous females (denoted by 'a'). See text for details. Note: scale bar=100μm.

Soma Size in the Facial Nucleus
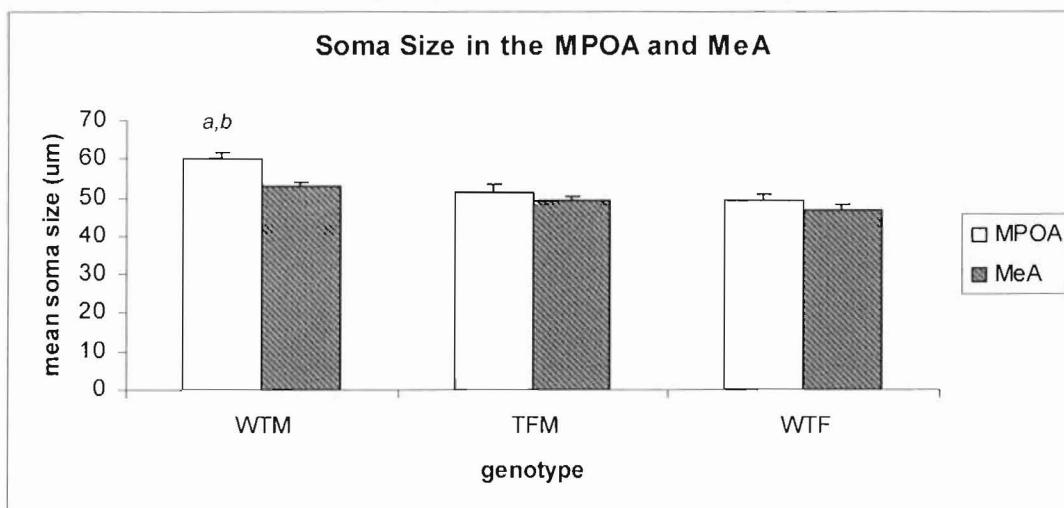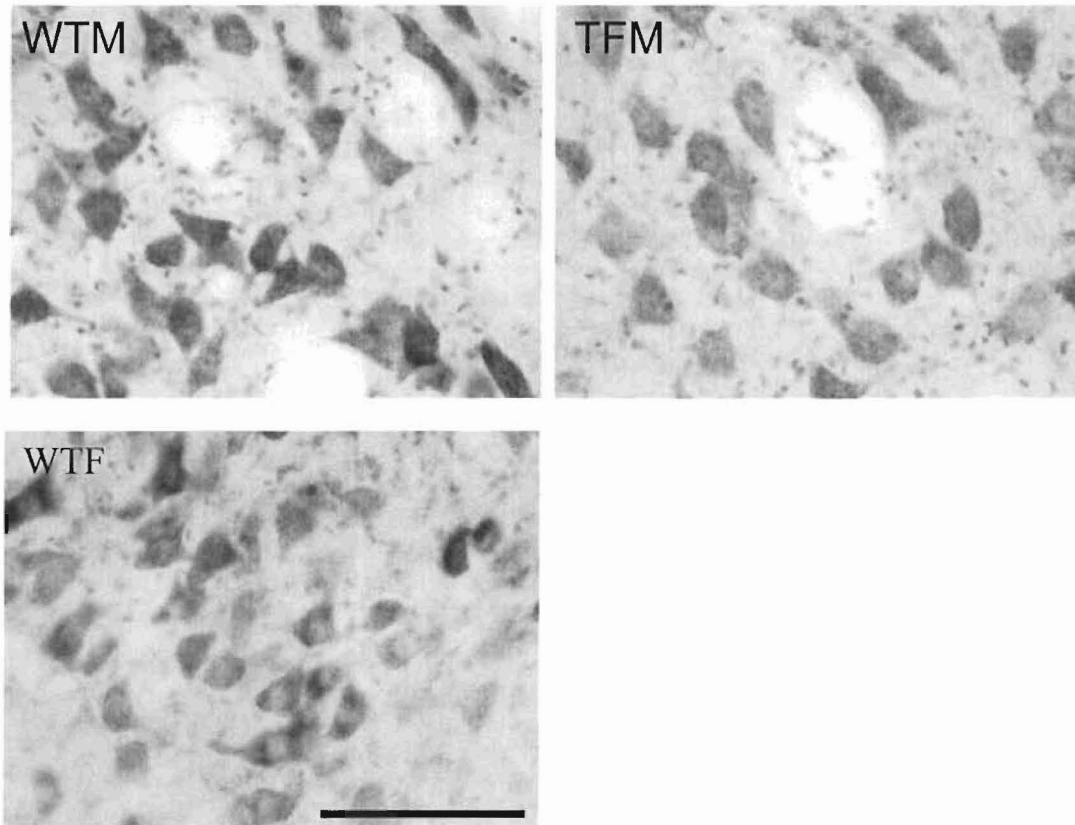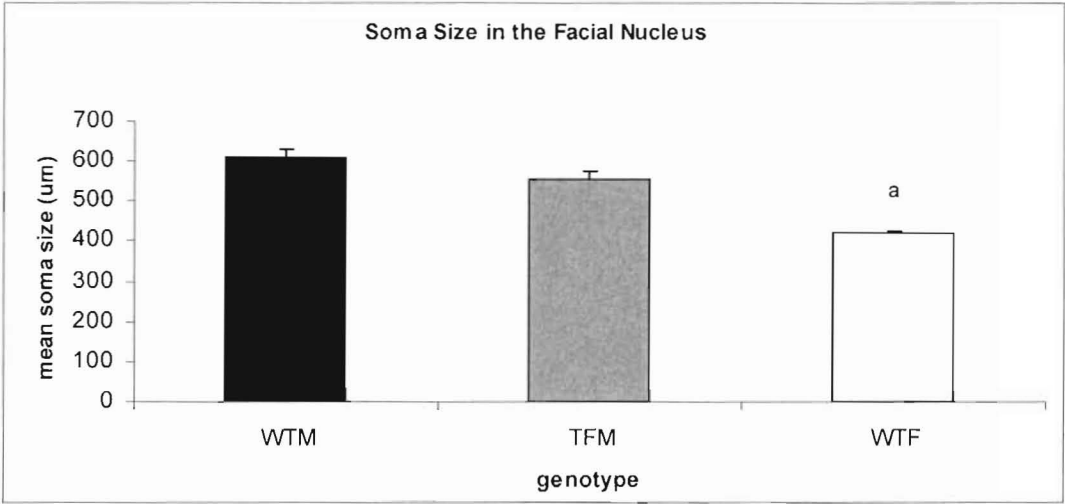
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CHAPTER 3 ELEVATED PLUS MAZE PERFORMANCE IN ANDROGEN-INSENSITIVE MALE RATS COMPARED TO WILD-TYPE MALES AND FEMALES

3.1 Abstract

Previous studies have shown that male rats carrying the testicular feminization mutation (TFM), an androgen receptor mutation which renders them insensitive to the actions of androgens such as testosterone, display sexual performance abnormalities. Because androgens can regulate anxiety-like behaviours, it is possible that the androgen receptor mutation may affect anxiety levels, and indirectly decrease the display of proper mating behaviour. Indeed, analysis of the adrenal glands reveals the zona reticularis is enlarged, as well, serum corticosterone levels have been observed to be elevated. We compared the performance of gonadally intact TFM rats with that of gonadally intact wild type (WT) males, and ovariectomized females given implants of estrogen followed by injections of progesterone or implanted with blank capsules and given injections of oil on two tests on the elevated plus maze (EPM). The EPM is an ethologically valid test of anxiety-like behaviour in rats. We observed the TFMs to display masculine performance on the EPM as there were no differences in terms of number of entries and time spent on the open arms compared to WT males. In terms of the percentage of time in the open arms, high serum levels of estrogen appeared to be anxiolytic, as TFMs, WT males, and blank treated females displayed a lower percentage compared to the estrous females. The results indicate the androgen mutation does not affect performance on the EPM and also suggests the lack of sexual performance is not due to increased anxiety levels.

3.2 Introduction:

Male rats carrying the testicular feminization mutation (TFMs) display feminine external genitalia (i.e., underdeveloped vagina and short anogenital distance) and a nipple line (Allison, Chan, Stanley, Gumbreck, 1971). This external female phenotype is due to end organ sensitivity to androgens caused by a mutation in the gene encoding the androgen receptor (Yarbrough, Quarmby, Simental, Joseph, Sar, Lubahn, Olsen, French, Wilson, 1990). Because the TFMs are chromosomally male and carry the SRY gene, expression of sry results in differentiation of the bipotential gonad into testes. In normal wild type (WT) males, testosterone expression spares the Wolfian system (made up of the male internal reproductive organs such as the epididymus, vas deferens, seminal vesicles, and vas deferens). Additionally, the testes also release another hormone, anti-Mullerian hormone, which causes regression of the female internal reproductive organs (such as the fallopian tubes, uterus, and inner vagina). Due to a lack of sry expression and androgenic signaling, the female internal reproductive phenotype develops. TFM affected males express sry, resulting in differentiation of the testes, however, due to the insensitivity of androgens, the Wolfian system degenerates, but due to the expression of anti-Mullerian hormone, the Mullerian system also degenerates (Stanley, Gumbreck, Allison, Eeasley, 1973). In TFM affected male rats, serum testosterone levels have been reported to be higher than in wild type (WT) males (Roselli, Sallisbury, Resko, 1987). While the TFMs do not display feminine sexual behaviour, unless castrated just after birth and injected with female typical hormones in adulthood, masculine sexual behaviour has been observed to be extremely variable (Olsen, 1979; Shapiro, Goldman, Steinbeck, Neumann, 1976; Beach and Buehler, 1977). In an attempt to understand the nature of the sexual

behaviour deficits, Olsen (1979) castrated TFMs in adulthood and treated them with either testosterone (T), or one of its two metabolites, 17 beta estradiol (E2) and dihydrotestosterone (DHT), alone or in combination. Maximal stimulation of male sexual behaviour appeared to be achieved following E2 treatment, whereas DHT alone did not have an effect. It was suggested that aromatization of T to E2 in the TFMs was adversely affected by the androgen receptor mutation, and this was the mediating factor in the lack of proper masculine sexual behaviour. However, several findings argue against this possible interpretation. Serum estrogen levels are reportedly higher in the TFMs compared to WT males (Roselli, Salisbury, Resko, 1987), the number of estrogen binding sites in the hypothalamus, an area critically important for the display of masculine sexual behaviour, appeared to be masculine (Olsen and Whalen, 1982), and while aromatase activity is compromised in the hypothalamus, activity was observed to be normal in the medial amygdala (Roselli, Salisbury, Resko, 1987). Thus, it seems estrogen should function to stimulate masculine sexual activity, however, this is not the case. One criticism of this study, though, was that no base line data regarding sexual activity was collected before gonadectomy and hormone replacement therapy (Olsen, 1979). Thus, because these animals were sexually naïve before being treated with the various hormone regimes, the results appear to be confounded with practice effects. Additionally, the sequence of hormone therapy was not counterbalanced and thus order effects may also be a potential confound. Despite these confounds, the androgen receptor mutation may potentially affect non reproductive behaviours, which may indirectly interfere with the TFMs to display proper sexual activity. Two observations suggest this may be the case: the adrenal glands, which release corticosterone from the zona

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reticularis of the medulla, are enlarged in the TFMs; subsequent analysis of corticosterone levels has revealed that serum values are elevated in the TFMs suggesting these mutants may be stressed.

Androgens contribute to the organization and activation of a wide variety of behaviours, many of which are sexually dimorphic. For example, male rats display greater frequency of juvenile play behaviour, and this depends upon the organizational actions of androgens during a critical developmental period (reviewed in Meaney, 1988). Androgen receptor activation also appears to be necessary for spatial performance; male rats outperform females in the Morris Water Maze, however, TFM affected males display only partially masculinized performance when compared to wild type (WT) males and females (Jones and Watson, 2005). In humans, reports of depression and anxiety appear to be sexually dimorphic, as well. Women report these emotional problems nearly twice as often as men do (Toufexis, Myers, Davis, 2006); rates of reporting anxiety also coincide with menopause in women (reviewed in Walf and Frye, 2006), further supporting a role of gonadal hormones.

Anxiety is often measured in rats via the use of the elevated plus maze (EPM). The apparatus used consists of two closed arms and two open arms, arranged in a plus pattern, and the whole apparatus is elevated off the ground. The open arms have been suggested to be anxiety inducing, as open spaces are thought to be anxiogenic in rodents, and as such, rodents prefer the closed arms. There also appears to be a sex difference in EPM behaviour, as estrous females have been reported to make more open arm entries and spend more time on the open arms than males, which tend to show an aversion to exploring this part of the EPM and spend most of their time in the closed arms (Johnston

and File, 1991). Castrated male rats display increased levels of anxiety-like behaviour on the elevated plus maze and open field, whereas decreased levels of anxiety-like behaviour were observed following injections with androgens, such as testosterone or dihydrotestosterone (Edinger and Frye, 2006). Interestingly, it has been postulated that androgens can decrease anxiety via conversion to GABAa receptor agonists (Aikey, Nyby, Anmuth, James, 2002). Exposure of male mice to females before placement into the EPM reduced levels of anxiety in the males as they displayed more open arm entries than non exposed controls; it has been postulated the anxiolysis may have been mediated via a testosterone surge which normally follows exposure to estrous females. The increased androgen substrate in the male mice was suggested to be reduced to other metabolites, such as androsterone and 3 alpha androstanedione, as this effect was replicated following injection of these substances, and in a very rapid manner, suggesting non genomic effects (Aikey, Nyby, Anmuth, James, 2002). Finally, blockade of the anxiolytic effects of testosterone was achieved with injection of the GABAa receptor antagonists, picrotoxin and bicucculine (Aikey, Nyby, Anmuth, James, 2002). However, a role for estrogen converging on the same receptor system has been proposed as regulating steroid mediated anxiolysis in the EPM. Female rats normally display fluctuations in the levels of estrogen and progesterone over the estrous cycle, and it has been observed that the greatest amount of anxiolysis is on the day of proestrous when serum estrogen levels are the highest. Female rats at this stage of the cycle made more open arm entries and spent more time on the open arms on the EPM compared to females at different stages of the estrous cycle (Frye, Petralia, Rhodes, 2000). Interestingly, the anxiolytic effects were also associated with increased concentrations of progesterone in

the hippocampus; progesterone is a known substrate of 5 alpha reductase, which can be

subsequently reduced to a metabolite, allopregnanalone (3α-hydroxy-5α-pregnan-20-one;

$3\alpha,5\alpha$-THP) a well known anxiolytic (Follesa, Biggio, Caria, Gorini, Biggio, 2004), with

GABAa receptor agonist activity (Frye, Petralia, Rhodes, 2000).

However, a direct role for estrogen-induced anxiolysis has been observed through

activation of the estrogen receptor beta (ER beta) isoform; intrahippocampal injection of

the ER beta selective agonist, diarylpropionitrile, into ovariectomized female rats

attenuated anxiety like behaviour on the EPM (Walf and Frye, 2006). Thus, there are at

least three known mechanisms of anxiolysis: two of which involve reduction of

androgens or estrogens to GABAa receptor agonists through the action of 5 alpha

reductase, or through direct interaction of estrogen with the ER beta isoform, which

presumably leads to anxiolysis through non genomic means.

In the current report, we used the EPM to assess the level of anxiety in male rats

carrying the testicular femininzation mutation (TFM) compared to gonadally intact wild

type (WT) males, or ovariectomized females implanted with estrogen (with subsequent

progesterone injections to bring them into behavioural estrous) or blank capsules.

Indexing the level of anxiety in the TFMs as either male- or female-like may give some

insight into whether or not the behavioural affective state of these animals may be

playing a mitigating role in their lack of sexual performance.

3.3   Methods

3.3.1   Animals

Sprague dawley wild type (WT) male (n=15), TFM affected males (n=19),

estrogen implanted (n=10; estrous), and blank implanted (n=13; non estrous) females

were obtained from our colony at Simon Fraser University. Animals were generated in our colony from females previously identified to be known carriers of the TFM mutation. Carrier females were mated with stud males obtained from the University of British Columbia. Offspring were weaned at 30 days of age, and at this time, were separated into WT male and mixed female/TFM cages based on phenotypic markers. WT males were identified based upon the appearance of a scrotal sac and large ano-genital distance. Unidentified daughters of known carriers (UDOCs) and TFMs can not be distinguished based on phenotypic markers; thus, we employed an assay to identify carrier females from WT females, and from TFMs based upon amplification of a region of the androgen receptor (details in Fernandez, Collado, Garcia Doval, Garcia-Falgueras, Guillamon, Pasaro, 2003). All animals were kept on a reverse 12:12 hr light/dark schedule with food and water available *ad libitum*.

3.3.2 Ovariectomies

At approximately 55-65 days of age, females were ovariectomized under inhalant (isofluorane) anesthesia and aseptic conditions. During surgery, 10 females were implanted with an interscapular subcutaneous 10mm silastic capsule (1.57mm internal diameter, 3.18mm outer diameter, 10mm in length; Dow Corning, Midland, MI) containing crystalline E2 (Steraloids, Chicago, IL), whereas 13 were given blank implants. The females were given post operative medication for pain and infection, and allowed to recover for at least 10 days. Four hours before behavioral testing, estrogen implanted females were subcutaneously injected with a 0.5 cc bolus of 500µg of progesterone (Steraloids, Chicago, IL) dissolved in corn oil whereas blank implanted females received oil only (0.5 cc). All surgical procedures conformed to the standards of

the Canadian Committee for Animal Care, and were performed under the supervision of an institutional veterinarian; the experimental protocol was subject to prior approval by the Simon Fraser University Animal Care Committee.

3.3.3 Elevated Plus Maze Apparatus

The elevated plus maze used in the current experiment was made out of laminated press board that was white in color. It consisted of 4 arms, 50cm long x 10cm wide, with 2 arms also containing 40 cm high walls enclosing the arms, and arranged in a cross shape; the whole apparatus was raised up off the ground by 62 cm. A schematic of the apparatus is displayed in figure 12.

3.3.4 Testing Procedure

WT males, TFMs, and females were tested, under red light illumination, in the EPM twice on separate days (one week apart). It has been recognized that a test/retest paradigm results in increased anxiety levels on the second day; performance on the first day may not produce reliable anxiety measures as rodents have a tendency to explore novel environments (Carobrez and Bertoglio, 2005). Each animal, chosen at random, was placed in the center of the apparatus, facing one of the closed or open arms (determined in a random fashion). The animals were allowed to explore the apparatus for a maximum of five minutes; following this, the animals were returned to their home cages. In between each test session, the apparatus was thoroughly cleaned with Quatricide. All tests were videotaped using a Sony DVD Handycam Camcorder (Sony, Canada) and analyzed on a PC computer by 2 observers blind to experimental conditions (inter rater reliability was 0.92). An arm entry was scored only when all four paws as

well as the base of the tail had passed the threshold of the arm. The number of closed and open arm entries was quantified, and a total of overall arm entries was calculated by adding these two values together. Additionally, we quantified the amount of time spent on the closed and open arms. From these values, we calculated a ratio of open arm entries to total entries (% entries into the open arms), and a ratio of the time spent on the open arms to the total time of the test (% time on open arms). These last two measures have been reported to be excellent indicators of anxiety behaviour on the elevated plus maze (Espejo, 1997).

3.4   Statistical Analyses

All analyses were carried out using SPSS 15.0 for Windows. Measures on the elevated plus maze were subjected to a one way analysis of variance (ANOVA) with alpha set at 0.05; where appropriate, *post hoc* comparisons were made using t-tests with a Bonferonni correction for multiple comparisons. Because previous studies have observed a sex difference in performance on the elevated plus maze (Johnston and File, 1991), with estrous females displaying less anxiety-like behaviour than males, we first compared the performance of these two groups on days one and two using a one-tailed T test with alpha set at 0.05. Additionally, reports suggest estrous females also display less anxiety-like behaviour than non estrous females (Frye, Petralia, Rhodes, 2000), and thus we also compared the performance of these two groups on days one and two using one-tailed T tests with alpha set at 0.05. Finally, it has been suggested that animals subjected to the elevated plus maze may display behavioural habituation with repeated testing (Espejo, 1997). Because the number of closed arm entries appears to represent purely motor activity (as found by a factor analysis; Cruz, Frei, Graeff, 1994), an analysis of this

measure may give some indication if the animals used in the current study habituated to the apparatus with repeated testing. We examined this by comparing the number of closed arm entries on day one to day two using a two way pair wise T test with alpha set at 0.05.

3.5 Results

3.5.1 Sex Difference Analysis

On day one, WT males and estrous females did not differ on any of the parameters quantified. However, sex differences emerged, in which males displayed greater anxiety-like behaviour, on day two of testing. The estrous females made more entries onto the open arms (t_{23}=2.59; p=0.0135), spent longer on the open arms (t_{23}=2.814; p=0.009), made a greater percentage of entries into the open arms (t_{23}=2.4; p=0.015), and spent a longer percentage of time in the open arms (t_{23}=2.8; p=0.009) compared to the males. However, the males spent longer in the closed arms compared to the estrous females ($t23$=2.86; p=0.0045). No differences were detected in terms of the number of closed arm entries (t_{23}=0.82; p=0.21) or the total number of entries made (t_{23}=1.5; p=0.08) between the two groups of animals.

3.5.2 Estrous Analysis

When we compare the performance of the estrous females to the non estrous females, the analyses suggested again that the estrous females displayed less anxiety. The estrous females made more entries onto the open arms ($t_{11.315}$=2.506, p=0.022), however, the non estrous females made more entries into the closed arms ($t_{19.53}$=2.151, p=0.016). The estrous females spent more time in the open arms ($t_{12.2}$, p=0.0135), whereas the non

estrous females spent more time in the closed arms (t_{21}=0.004) in the five minute test.

The estrous females also spent a larger percentage of the test time on (t_{13}=2.466,

p=0.014), and made a larger percentage of entries onto ($t_{12.23}$=2.502, p=0.013), the open

arms compared to the non estrous females. We next compared WT males and females to

the performance of the TFM affected males on the elevated plus maze.

3.5.3 Analysis of Anxiety Behaviour Between TFMs, WT males, and females (estrous and non estrous)

3.5.3.1 I) Day One

The analysis of performance on day one did not reveal any statistically significant results

(see Figure 13).

3.5.3.2 II) Day Two

The ANOVA revealed that there was a significant effect of genotype on the number of

open arm entries made ($F_{3,53}$=6.061, p=0.001), the number of closed arm entries made ($F

_{3,53}$=3.562, p= 0.021), the total number of entries made ($F_{3,53}$=2.979, p=0.04), the total

time spent on the open arms ($F_{3,53}$=5.212, p=0.003), total time spent on the closed arms

($F_{3,53}$=3.648, p=0.018), the number of open arm entries as a ratio of the total entries into

any arm ($F_{3,53}$=4.178, p=0.01), and the percentage of time spent on the open arms as a

ratio of the overall time in the EPM ($F_{3,53}$=5.212, p=0.003). *Post hoc* tests revealed the

following pattern of results.

Open arms- The data suggest the TFMs, WT males, and non estrous females

performed similarly in terms of the number of open arms made when compared to the

estrous females (see figure 13). The *post hoc* analysis revealed the TFMs, WT males,

and non estrous females all made fewer entries onto the open arms compared to the

estrous females (TFM vs estrous female, p=0.002; WT male vs estrous female, p=0.001; Non estrous female vs estrous female, p=0.006), but no differences were detected between the TFMs, WT males, and non estrous females (p=0.449). The interpretation of these results, that estrogen produces anxiolytic effects, is in agreement with previous studies (Johnstone and File, 1991; Walf and Frye, 2006).

Closed arms- In terms of the number of closed arm entries, the *post hoc* analysis revealed some equivocal results (see figure 13). When the number of open arm entries is compared to the TFMs, the WT males and estrous females do not differ (vs. WT males, p=1.00; vs. estrous females, p=1.00); however the non estrous females made more closed arm entries than the TFMs (p=0.015). Additionally, WT males, estrous and non estrous females do not differ in the number of closed entries made (WT males vs estrous females, p=1.00; WT males vs non estrous females, p= 0.426; estrous females vs non estrous females, p= 0.24).

Total Arm Entries- While the omnibus *F* test was significant at the 0.05 level (see above), no *post hoc* comparisons, corrected for family wise error rate, were significant (see figure 13).

Time in the Open Arms- The *post hoc* analysis of time spent in the open arms suggests the estrous females spent more time in the open arms compared to the other groups (vs TFMs; p=0.05; vs WT males, p=0.003; vs non estrous females, p=0.01) (see figure 14). No other comparisons were significant (WT males vs TFMs, p=1.00; WT males vs non estrous females, p= 1.00; TFMs vs non estrous females, p=1.00). Again, the data suggest the high estrogen levels reduced anxiety in the estrous females compared to the other groups.

Time in the Closed Arms- The *post hoc* analysis suggests that decreased serum

estradiol affected the number of closed arm entries made as the non estrous females made

more compared to the estrous females (p=0.018) (see figure 14). This interpretation only

applies to the females as the TFMs and WT males did not differ from both the estrous

and non estrous females (TFMs vs WT males, p= 1.00; TFMs vs estrous females, p=

0.797; TFMs vs non estrous females, p= 0.319; WT males vs estrous females, p=0.117;

WT males vs non estrous females, p= 1.00).

Percentage of Open Arm Entries- The *post hoc* analysis of the number of open

arm entries as a ratio of the number of total arm entries (i.e., % open arms) also suggested

some equivocal results (see figure 15). The data are consistent with an effect of estrogen

on this parameter, but only if the TFMs are not considered. That is, the estrous females

displayed a higher percentage of open arm entries compared to the WT males (p=0.043)

and non estrous females (p=0.038), with WT males behaving similarly to the non estrous

females (p=1.00). However, when the TFMs are considered in comparison with the other

groups, no differences are detected (vs WT males; p=0.245; vs estrous females, p=1.00;

and vs non estrous females p=0.212).

Percentage of Time on the Open Arms- The *post hoc* analysis of the time spent on

the open arms as a ratio to the total test duration (i.e., 300 seconds) also revealed an effect

of high serum estrogen on performance (see figure 15). The estrous females displayed a

larger percentage of time on the open arms compared to the TFMs (p=0.05), WT males

(p=0.003), and non estrous females (p=0.01). However, the TFMs, WT males, and non

estrous females did not differ from each other (WT males vs TFMs, p=1.00; WT males vs

non estrous females, p=1.00; TFMs vs non estrous females, p=1.00).

Analysis of Locomotor Activity- In order to discern if repeated testing in the elevated plus maze produced behavioural habituation, we compared the number of closed arm entries from day one with day two. The number of closed arm entries made by the WT males (t_{14}= 2.617, p=0.02), estrous females (t_9=3.674, p=0.005), and non estrous females (t_{12}= 2.578, p= 0.024) increased from test one to test two, however, the number of closed arm entries did not differ in the TFMs (t_{18}=0.589, p= 0.563) (see figure 16). For habituation to be claimed, the number of closed arm entries should be observed go down from test one to test two. However, this was not observed in the current report as WT males, estrous females, and non estrous females actually displayed increased motor activity with repeated testing; the TFMs, though, made similar amounts of closed arm entries.

3.6   Discussion

We observed several patterns in the behaviour of the animals used in the current report. High serum estradiol in females was associated with more entries into, and spent more time on, the open arms compared to the other groups. An effect of estrogen on the amount of time spent in the closed arms was only observed in the females, as the non estrous females spent more time in the closed arms (a sign of anxiety) compared to the estrous females. We interpret these data in this manner as there were no differences between the amount of time spent in the closed arms in the TFM affected and WT male groups; previous reports indicate the TFMs contain higher serum estradiol than the WT males (Roselli, Salisbury, and Resko, 1987).

Regarding the anxiety levels of the TFMs, based upon the analysis of the percentage of time spent on the open arms, it appears these mutants display masculine

anxiety-like behaviour, as the TFMs performance did not differ from the WT males. Percentage of time spent on the open arms has been suggested to be a relatively pure indication of anxiety (Cruz, Frei, Graeff, 1994; Rodgers and Johnson, 1995). The percentage of open arm entries made has also been interpreted as a good indicator of anxiety; in the current report, we did find some equivocal results in that the estrous females made a larger percentage of open entries compared to the WT males and non estrous females, however, the TFMs did not differ from any of the groups. The percentage of open arm entries made as a function of total entries uses the absolute number of open arm entries in the calculation of this measure; Cruz, Frei, and Graeff (1994) report that the absolute number of open arm entries is a mix of anxiety and motor activity. This makes sense as there is a motor component to this behaviour in that the animals must move to the open arms to make an entry. Thus, the data regarding the percentage of entries in to the open arms in the current report may indicate that the TFMs display some anomalous motor activity that cannot be characterized as either male- or female-like. This interpretation is supported by the analysis of the number of closed arm entries; which has been characterized as a relatively pure measure of motor activity (Cruz, Frei, Graeff, 1994). While the WT males, estrous, and non estrous females all increased their motor behaviour from test one to test two, the TFMs animals were the only group observed not to change their motor behaviour.

Our results are not in agreement with those of Zuloaga, Jordan, Breedlove (2006) who also examined anxiety in the TFM affected male rats. They reported that TFMs made fewer entries into the open arms than the closed arms and made fewer line crossings in the open field compared to WT males suggesting the androgen receptor

mutation resulted in increased anxiety. Possible strain differences could account for the disparity of results as the TFM mutation in our lab is on the Sprague Dawley background, but is on the Long Evans background in the Breedlove/Jordan lab. However, the type of mutation carried by these animals is exactly the same in that there is a change of arginine to glutamine at amino acid 734 (Yarbrough, et al., 1990). Strain differences have been reported between Sprague dawley and Long Evans in terms of acoustic startle response, which has also been used as a measure of anxiety (Acri, Brown, Saah, Grunberg, 1995).

That the TFMs did not display any difference in anxiety-like behaviours compared to the WT males suggests that these mutants are masculinized for this function. As mentioned (see introduction), the anxiolytic effects of androgens can possibly be achieved in different ways. For example, the reduction of testosterone to androsterone or 3 alpha androstanedione, via 5 alpha reductase, may result in anxiolysis via interactions with the GABAa receptor channel. These significant anxiolytic effects are only observed if there is increased substrate (i.e., testosterone or progesterone) to be reduced to these GABAa agonists. The TFMs have been reported to display increased levels of serum testosterone (Roselli, Salisbury, Resko, 1987), compared to WT male littermates, suggesting ample prohormone is available for reduction or aromatization. Thus, given these observations, one would expect the TFMs to display decreased levels of anxiety in the elevated plus maze; however, this was not the case. One possibility that could account for this observation is that production of the gene encoding the 5 alpha reductase isozyme has been reported to be mediated by androgen receptor activity (George, Russell, Wilson, 1991). Thus, as TFMs are insensitive to the actions of testosterone, whereby the mutated androgen receptor appears to not have transcriptional capabilities when activated

with physiological levels of androgens (however, see Naess, Haug, Attramadal, Aakvaag, Hansson, French, 1976), one would expect the levels of 5 alpha reductase to be much lower in the TFMs compared to WT males. Given the lack of masculine differentiation of the external phenotype during development, which is regulated by DHT and 5-alpha reductase, the most parsimonious explanation would be that the TFMs are deficient in this enzyme. Additionally, it has been reported that serum DHT is less than half the amount in the TFM compared to males (Roselli, Salisbury, Resko, 1987). If indeed it is the case that 5 alpha reductase is deficient in the TFM, then anxiolysis by reduction of progesterone to metabolites with GABAa agonist actions are also not likely, as they too would require sufficient amounts of the 5 alpha reductase enzyme. This then leaves the possibility that anxiolysis could be achieved through interaction of estrogen with the ER beta isoform. It has been reported that infusion of antisense oligodeoxynucleotidies to ER beta decreased the number of open arm entries made compared to controls injected with a scrambled sequence suggesting a lack of ER beta signaling increased anxiety (Walf, Ciriza, Garcia-Segura, Frye, 2007).

As mentioned (in the introduction), it has been previously reported that estrogen binds normally in the hypothalamus of TFM affected mice, suggesting the estrogen receptor system is not affected by the androgen receptor mutation (Olsen and Whalen, 1982). However, this study was conducted before the discovery of ER beta, which is the product of a gene on a different chromosome as estrogen receptor alpha (Mosselman, Polman, Dijkema, 1996). Both receptor isoforms have similar DNA binding domains and affinities for E2 (Kuiper, Enmark, Pelto-Huikko, Nilsson, Gustafsson, 1996). ER alpha has been observed to be decreased whereas ER beta has been observed to be

increased following estrogen treatment (Osterlund, Kuiper, Gustafsson, Hurd, 1998), suggesting differential effects upon the production of the ER isoforms via estrogen. Thus it appears that the previously observed high endogenous levels of estrogen in the TFM (Roselli, Salisbury, Resko, 1987) would likely up regulate ER beta production. Given that ER beta activation can have anxiolytic effects when activated in the hippocampus (Walf and Frye, 2006), it would seem reasonable to suggest the TFMs should have displayed less anxiety than the WT males in the EPM. Again, though, this was not observed in the current report.

While speculative, it can be suggested that perhaps both the androgen and estrogen receptors cooperate to produce steroid mediated anxiolysis. If true, the in the TFMs, the loss of androgen receptor signaling may have increased anxiety, however, a subsequent ER beta mediated anxiolysis may have compensated for the lack of androgenic signaling. To date, though, ER beta levels have not been examined in the TFMs.

3.7 Summary and Conclusions

We have observed the TFMs in the elevated plus maze and compared their performance to WT gonad intact males and ovariectomized females artificially brought into estrous or left non cycling. Our results suggest the TFMs display masculinized levels of anxiety, and thus we conclude that sexual performance in mating tests is specific to reproductive behaviour and not to increased levels of anxiety.

Figure 12 Schematic of the Elevated Plus Maze apparatus

50cm

62cm

10cm
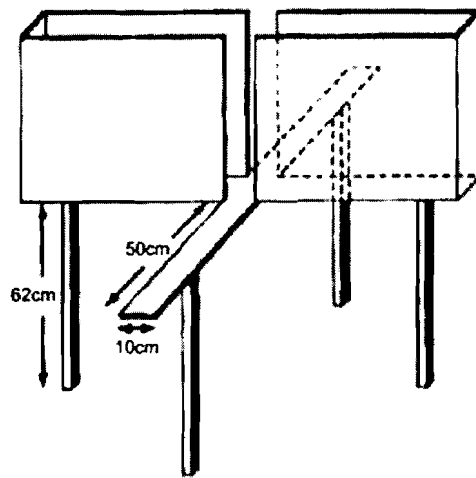
Figure 13 Day one and Day Two Elevated Plus Maze performance in wild type (WT) males, males carrying the testicular feminization mutation (TFM), estrous females, and non estrous females. Note: * in Day Two, open arm entries= estrous female different from all groups (p,0.05; see text for description). * in Day Two, closed arm entries= Non estrous females different from TFMs, only (p=0.015; see text for description).

Day One

Day Two

Number of Open Arm Entries

Open Arm Entries

*

mean entries

TFM   WT Male   Estrous Female   Non Estrous Female
genotype

mean entries

TFM   WT Male   Estrous Female   Non Estrous Female
group

Number of Closed Arm Entries

Close Arm Entries

*

mean entries

TFM   WT Male   Estrous Female   Non Estrous Female
group

mean entries

TFM   WT Male   Estrous Female   Non Estrous Female
group

Number of Total Arm Entries

Total Arm Entries

mean entries

TFM   WT Male   Estrous Female   Non Estrous Female
group

mean entries

TFM   WT Male   Estrous Female   Non Estrous Female
group

Figure 14 Time spent in the open and closed arms on Day One and Day Two. Note: * Day Two, time in the closed arms= estrous females spent the most time in the closed arms compared to all other groups (see text for details). * in Day Two, time in the closed arms= Non estrous females spent more time in the closed arms compared to the estrous females (see text for details).
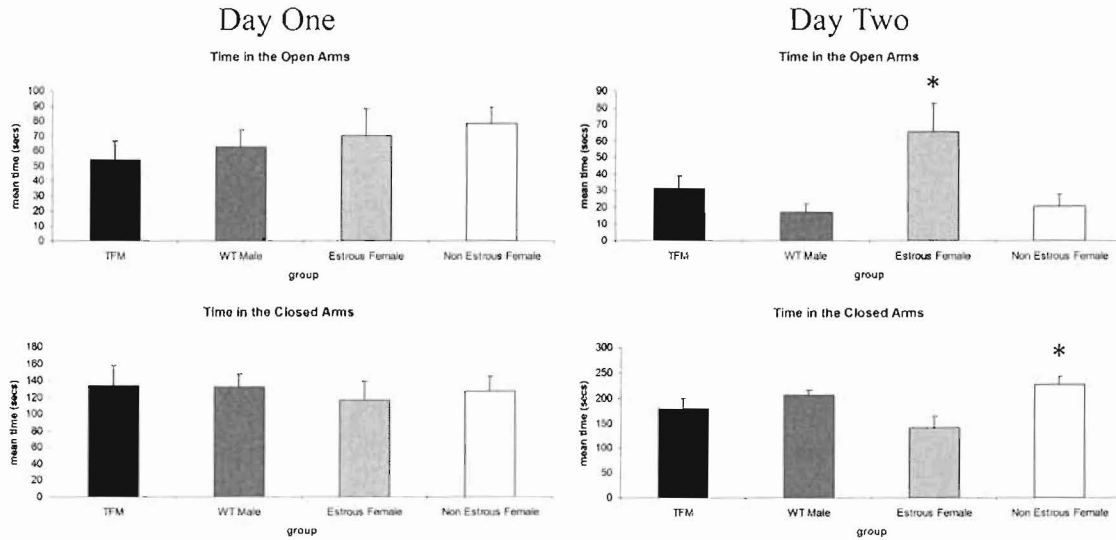
Figure 15 Percentage of Open Arm Entries and Percentage of Time spent in the Open Arms. Note *= Estrous females different from Non Estrous females (see text for details). **= Estrous females different compared to WT males (see text for details). Alpha=estrous females different than all other groups (see text for details).
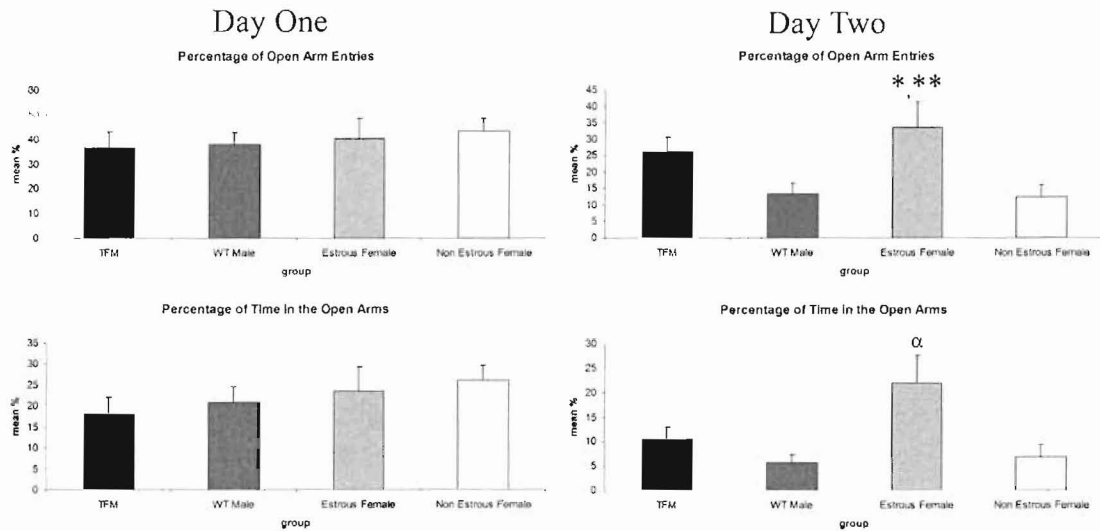
Figure 16 Analysis of Motor Activity (via number of Closed Arm Entries; see text for details).   Note:
* denotes significant difference between Days One and Two .

Closed Arm Entries
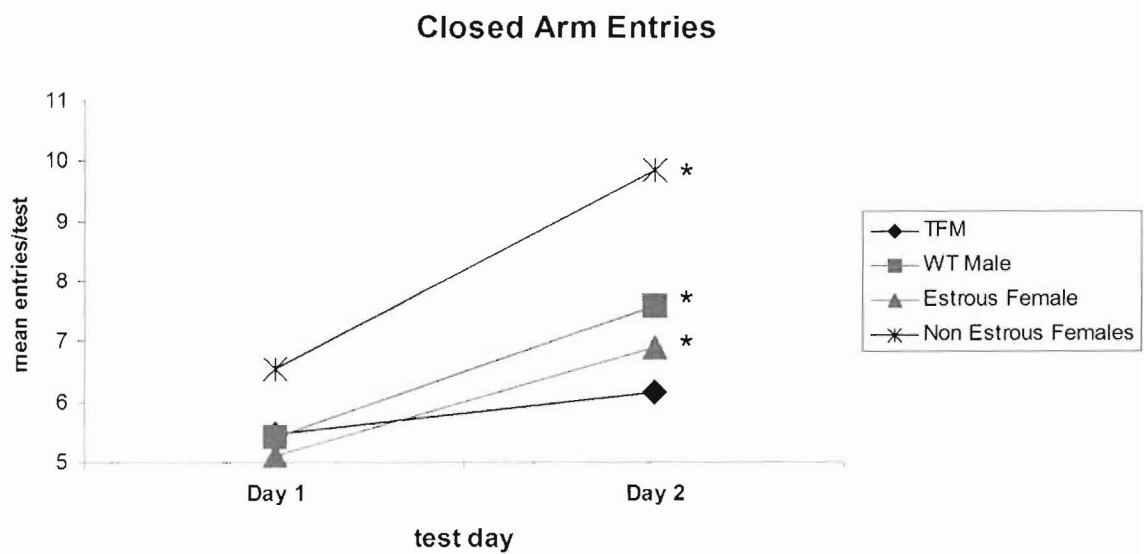
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CHAPTER 4 PARTNER PREFERENCE AND MOUNT LATENCY ARE MASCULINIZED IN ANDROGEN INSENSITIVE RATS.

4.1   Abstract

The preference for an estrous female over a non-cycling female rat and the display of short mount latencies have both been used as indicators of the motivation of male rats to engage in sexual behaviour. Partner preference was assessed two different ways in rats carrying the testicular feminization mutation (TFM), and compared to wild type (WT) males in one version of this paradigm, and WT females and males in another version. Additionally, mount latency was quantified in the TFMs and compared to WT males in order to assess arousal levels, as this has not been previously reported. When presented with an estrous or non estrous female, both WT males and TFMs preferred to spend more time with the estrous female. Similar results were obtained when TFMs and WT males were presented with a WT male or an estrous female; however, sexually receptive females preferred to spend time with the WT male. As had previously been reported, TFMs displayed sexual performance deficits, however, mount latencies were in the male range. Given that the TFMs reliably choose to spend time with the estrous female in the partner preference tests, and that they display normal arousal levels, the data suggest the motivation to engage in sexual behaviour is masculine in the TFMs and that possession of a functional androgen receptor is not crucial in these behaviours.

4.2　Introduction

The motivation to engage in sexual behaviour is often assessed via the partner preference paradigm. In such tests, the experimental male is presented with a choice between two different stimuli (an estrous female vs a sexually active male; or an estrous female vs an anestrous female), and the proportion of time the test subject spends with the receptive female serves as an index of sexual motivation. The preference of sexually experienced males for estrous females reportedly depends upon androgens (Everitt, 1990), as long-term castrates (which preferred the estrous female in baseline pre-gonadectomy tests) spend more time with a female not in estrus (Harding and McGinnis, 2004). What is more, when given a choice between the soiled bedding from an estrous female or from a sexually active male, gonadally intact male rats prefer to investigate the bedding from the estrous female (Bakker et al., 1994). Sexual arousal/motivation can also be inferred from the latencies to display certain sexual behaviours such as mounts and intromissions (however, see Everitt, 1990 for discussion). Animals highly sexually aroused express this internal state by beginning to display mounting activity sooner than animals not as aroused. This also appears to depend upon gonadal steroids, as neonatally androgenized females will begin to mount and display intromission-like behaviour with a similar latency to males; this behaviour is enhanced if the females are also treated in adulthood with testosterone (Sachs, Pollak, Krieger, Barfield, 1973).

Testicular androgens such as testosterone can be converted to estrogen *de novo* in cells that contain the enzyme, aromatase. Evidence indicates that estrogens, acting upon the alpha isoform of the estrogen receptor, are important for the organization and activation of masculinized female-oriented partner preference, but not mounting

behavior. Prenatal or neonatal inhibition of estrogen synthesis, via the use of aromatase inhibitors, reduces a male rat's partner preference for an estrous female (Brand et al., 1991). Likewise, mice homozygous for a mutation in the cyp19 gene (aromatase knockout (ArKO) mice) did not display the normal male preference for an ovariectomized, estrogen primed female mouse (Bakker et al., 2002), and displayed longer latencies to begin mounting activity (Honda et al., 1998). Similar deficits in partner preference were observed in the estrogen receptor alpha knock out mouse (ERαKO) (Wersinger and Rissman, 2002), however, parameters such as mount latency were left intact following gene deletion (Ogawa et al., 1997).

The gene encoding the androgen receptor (AR) resides on the X chromosome. A mutation in this gene has been described in rats (Allison et al., 1971) in which a single base mutation in the steroid binding domain results in a substitution of the amino acid glutamine in place of an arginine residue (amino acid residue 734) in the translated protein (Yarbrough et al., 1990). The arginine is highly conserved among the steroid receptor superfamily and may be important for phosphorylation, and thus activation, of the bound androgen receptor. Consequently, male rats hemizygous (i.e., $X^{tfm}Y$) for the AR mutation (tfm-affected male; TFMs), display normal affinity, but a decreased binding capacity, for androgens as assessed on kidney cytosol extracts (Yarbrough et al., 1990). As a consequence of this end-organ insensitivity to androgens, TFMs display an external feminine phenotype, including a shallow vagina, intact nipple line, and short (i.e. feminine) anogenital distance, but have internal testes that are androgen secretory (Roselli et al., 1987). Additionally, these animals reportedly exhibit none to very low levels of masculine sexual behaviour (Ohno, Geller, Young Lai, 1974; Shapiro et al.,

1976; Beach and Buehler, 1977). Olsen (1979) concluded that sexual behaviour can be activated by injection of testosterone or estrogen into gonadectomized TFMs, however, dihydrotestosterone was ineffective. Thus, it was suggested that the lack of sexual performance in the TFMs may be due to a lack of conversion of testosterone into estrogen. Indeed, aromatase, which is AR regulated in some tissues, has been reported to be decreased in the TFM medial preoptic area (MPOA) but not in the basolateral and medial amygdala (MeA) (Roselli et al., 1987). However, reports indicate that TFM rats (Olsen and Whalen, 1982) and mice (Fox, 1975) have normal amounts of estrogen binding in the hypothalamus and preoptic area, and that serum estrogen concentrations in rats are approximately double the amount in TFMs compared to WT male littermate (Roselli, Salisbury, Resko, 1987). Thus, if sexual behaviour solely relied upon estrogen receptor activation, the TFMs should not display the types of performance deficits that have been previously reported (Beach and Buehler, 1977; Shapiro et al., 1976). Perhaps other factors are affecting the ability of these animals to perform sexually.

Masculine sexual behaviour in rodents is often dichotomized into appetitive and consumatory components. Consumatory sexual behaviours include mounts, intromissions, and ejaculations; these are referred to as the performance aspects of sexual behaviour. Appetitive sexual behaviours include those behaviours necessary to make sexual contact more likely and include such behaviours as ultrasonic vocalizations, anogenital investigations, and pursuit behaviours; these behaviours are sometimes referred to as precopulatory or courtship behaviours. There are also behaviours displayed by the male rat that are thought to indicate sexual arousal; these include the latencies to achieve mounts, intromissions, and ejaculations. Thus, a sexually aroused and

experienced male rat may begin mating (i.e., by displaying mounts) with a shorter latency than one that is not aroused or has never had sexual contact.

The studies on TFM sexual behaviour mentioned above have assessed only the performance aspects of mating in these mutant animals (such as number of mounts, intromissions, and ejaculation), whereas precopulatory behaviours have never been assessed in TFM affected male rats (however, see Bodo and Rissman, 2007 for mice). Given the importance of appetitive behaviours for mating, deficits in copulation may be due to a decreased motivation of the TFMs to engage in sexual activity. The level of sexual motivation in TFMs using two different partner preference tests was assessed in the current report. The first one involved a two choice stimulus test in which the animals were presented with a gonadectomized female artificially induced into behavioural estrous, or with a gonadectomized, anestrous female rat. The second test involved presenting the TFMs with either a sexually active male or an estrous female. Additionally, sexual activity in TFMs such as the latency to begin mounting behaviour was assessed and compared to wild type male littermates as this has not previously been reported for TFM rats. Assessment of mount latency and partner preference using the TFM rat may give insights into the role the AR plays in the organization and activation of these aspects of sexual behaviour.

4.3   Methods

4.3.1   Generation of TFM-affected males:

Using a polymerase chain reaction based assay (Fernandez et al., 2003) females from our TFM colony that were heterozygous carriers of the testicular feminization mutation (i.e., carrier females, $X^{tfm}X$) were identified. These carriers (n=4) were mated with Sprague

Dawley stud males obtained from a breeding colony at the University of British Columbia, Canada. Pups were weaned at approximately 30 days of age and divided into separate cages based on phenotype; animals with a long anogenital distance and scrotal sac were classified as males, whereas animals with a short anogenital distance and no scrotal sac were classified as females. However, because TFMs, carrier females, and wild type females are practically indistinguishable from each other based on these phenotypic characteristics, the PCR based assay was used to further separate their offspring. Once the TFMs and WT males had been positively identified, they were housed by genotype and 2-4 animals per cage. All animals in this study were kept on a reversed 12hr light/12hr dark cycle, with food and water available *ad libitum*.

4.3.2 Stimulus Females:

For tests of sexual behaviour and partner preference, 17 Sprague Dawley females, approximately 50 days of age were ovariectomized under inhalant (isoflurane) anesthesia and aseptic conditions. The females were given post operative medication for pain and infection, and allowed to recover for at least 10 days. On the day of surgery, 13 were implanted with a 10mm silastic capsule (1.57mm internal diameter, 3.18mm outer diameter, 10mm in length; Dow Corning, Midland, MI) containing crystalline estrogen (Steraloids, Chicago, IL), whereas the other 4 females were implanted with capsules containing nothing. On test days, the estrogen implanted females were subcutaneously injected with a 0.5 cc bolus containing 500µg of progesterone (Steraloids, Chicago, IL) dissolved in corn oil 4 hours before behavioural testing, whereas the blank females were injected with a bolus of 0.5 cc of the vehicle, corn oil. All surgical procedures conformed to the standards of the Canadian Committee for Animal Care, and were performed under

the supervision of an institutional veterinarian; the experimental protocol was subject to prior approval the Simon Fraser University Animal Care Committee.

4.3.3 Partner Preference Tests

4.3.3.1 Experiment 1- Choice between an Estrous female or a non estrous female

All testing was done during the dark phase in the colony room and commenced 2 hours after lights out under red light illumination. At approximately 75-90 days of age, TFMs (n=10) and wild type males (WT males; n=9) were placed in the partner preference apparatus for approximately 15 minutes without the stimulus females in order to familiarize themselves with the testing equipment. The partner preference apparatus was modeled after (Everitt, 1990) with minor modifications (figure 17). Constructed from 0.5 inch laminated press board that was white in color, the apparatus contained three chambers in total. Two runway chambers (which housed the stimulus females), side by side, were 11.5 inches wide and 39.5 inches long, with a third neutral chamber being 7.5 inches wide and 23.5 inches long.

On test days, stimulus females (i.e., an estrous female and a non-estrous female) were tethered to the back of the runway chambers by a string tied to an animal jacket (Harvard Apparatus, St. Laurent, Quebec); the jackets did not inhibit the movement of the females at all and they appeared to behave normally after an initial acclimatization period. For example, the estrous females were observed to display normal proceptive behaviours such as hop-darting, ear wiggling, as well as receptive behavior, such as lordosis, when properly stimulated by the experimental animals. The apparatus was thus baited with an estrous female in one runway chamber and an anestrous female in the other. The order of baiting was counterbalanced between sessions.

Both TFMs and WT males were tested once in the partner preference apparatus when they were sexually naïve in order to obtain baseline partner preference data. They were subsequently given sexual experience and then tested again in the partner preference apparatus. The rationale for observing the TFMs sexual behaviour was to ensure that they did indeed display sexual performance deficits as has been previously reported (Beach and Buehler, 1977). On the test following sexual experience, two of the WT males were observed to investigate the tether of the non estrous female for the entire five minute test; because of this, all animals were rerun and the data of both groups was pooled and then averaged. Statistical analysis (see below) was run on the averaged time of test two and test three. On the day of testing, the experimental subjects were first placed in the neutral area for each of the five minute tests and allowed to freely explore all of the chambers. Once five minutes had elapsed, the TFMs or WT males were removed and placed back into their respective home cages and the test apparatus was thoroughly cleaned before the next experimental animal was tested.

4.3.3.2   Experiment 2- Choice between a wild type male or an estrous female

For this version of the partner preference test, a sexually active gonadally intact male or a gonadectomized female artificially brought into estrus via hormone priming were tethered to the back of the apparatus by the animal jackets used in the previous tests. Again, the jackets did not bother the animals in any way, as both were observed to display gender specific sexual behaviours during the actual tests. The order of baiting was also counterbalanced between sessions. A new sample of WT males (n=7), TFMs (n=7), and estrous females (n=7) were utilized for this test. Experimental animals were placed in the neutral part of the partner preference apparatus and allowed to freely

interact with the 'stimulus' animals for a maximum of five minutes. The animals were then removed, placed back in their respective home cages, and the apparatus was thoroughly cleaned before the next animal was tested. Animals were tested in random order.

All tests were videotaped using a Sony DVD Handycam Camcorder (Sony, Canada) and analyzed on a PC computer by an observer blind to experimental conditions. The length of time the test animal spent with each of the stimulus animals (defined as having all four paws in the chamber), as well as the amount of time spent in the neutral area, was quantified.

4.3.4 Sexual Behavior Tests

For tests of sexual behaviour, TFMs and WT males (the same animals utilized for *Experiment 1*; see above) were placed in 10 gallon glass fish tanks (52x25x30 cm, Hagen, Richmond, BC) containing corn cob bedding for a 5 minute acclimatization period. Following this, ovariectomized and steroid primed females were introduced and the timer was started. The experimental animals were given three tests in total, each lasting 30 minutes, and conducted once per week. Frequencies and latencies to mount, intromit, and ejaculate were quantified. Given that the TFMs have a feminine external phenotype, some clarification of their masculine sexual behaviour is needed. In the TFM male, mounting behaviour is identical to that of a WT male; the animal positions himself behind the stimulus female (displaying lordosis), clasps the female with his forepaws, and he may display pelvic thrusting behaviour. Intromissions and ejaculations displayed by the TFMs are also very similar to behaviours observed in WT males, even though the TFMs do not have a penis. For intromission-like behaviour, TFMs mount the lordosing

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female and display multiple pelvic thrusts, terminating with a single deep thrust and rapid backward dismount, as if the penis has been inserted into the vagina. Likewise, TFMs' ejaculation-like behaviour involves a final deep pelvic thrust (similar to an intromission) but with an extended clutching of the female, as well as back muscle twitching, and lacks the backward springing dismount that characterizes an intromission. In other words, the display looks exactly like an ejaculation from a WT male. Following ejaculation like behaviour, TFMs also display a post ejaculatory interval like that of a WT male; this is characterized by immobility, a general lack of interest in the stimulus female, and emission of a 22 kHz post ejaculatory ultrasonic vocalization (unpublished observations).

4.4 Statistical Analyses

All statistical analyses were carried out using SPSS (version 14.0) for Windows. Independent samples T-tests were employed to determine if TFMs and WT males differed in the amount of time spent in each chamber in *Experiment 1*; a one-way analysis of variance (ANOVA) was utilized to test for possible differences in time spent in each chamber in *Experiment 2* with genotype as the independent factor; *post hoc* analysis was made using Fisher's least significant difference (LSD) statistic. To determine partner preference (i.e., amount of time spent with the stimulus animals or in the neutral zone) within each genotype, a one way ANOVA was utilized (for both Test A and B), where appropriate, *post hoc* comparisons were made using Fisher's LSD. T tests were used to analyze sexual behaviour. For all statistical tests, alpha was set at 0.05.

4.5   Results

Overall, TFM and WT males showed highly comparable patterns of preference for estrous females in both *Experiment 1* (a and b) and *Experiment 2*. For *Experiment 2*, the estrous females preferred to spend more time with the sexually active male over the estrous (stimulus) female. Thus, the data suggest the TFM affected male is fully masculine in terms of partner preference, and thus the motivation to engage in sexual behaviour is intact despite a mutated androgen receptor.

4.5.1   Partner Preference Experiment 1a and 1b: Estrous and non estrous females used as stimulus animals

4.5.1.1   Experiment 1a: Sexually Naïve

Figure 18 displays the mean time (+/- SEM) spent in each of the chambers for the WT males and TFMs. Student's T test revealed that the TFMs and WT males did not differ in their behaviour in the partner preference apparatus in terms of the amount of time spent in each chamber (TFM vs WT male with the estrous female: $T_{18}=0.58$; 2 tailed significance=0.569; TFM vs WT male with non estrous female: $T_{18}=0.113$; 2 tailed significance=0.911; TFM vs WT male in neutral zone: $T_{18}=1.39$; 2 tailed significance=0.181).

WT males: On the first partner preference test, an analysis of variance (ANOVA) revealed an overall difference in the amount of time spent in each of the chambers for the WT males ($F_{2, 24}= 13.9$, $p<0.001$), with a *post hoc* analysis revealing that these animals preferred to spend more time with the estrous female versus the non-estrous female ($p=0.003$) or in the neutral zone ($p<0.001$) (see figure 19). The amount of time spent with the non estrous female did not differ from time spent in the neutral zone ($p=0.081$).

TFM affected males: For the sexually naïve TFMs, an ANOVA revealed a significant overall difference for time spent in each of the three chambers ($F_{2, 27}$= 10.143, p=0.001); the *post hoc* analysis revealed the TFMs preferred to spend more time with the estrous female over the non estrous female (p=0.035) (see figure 20). Additionally, more time was spent in the chamber containing the estrous female versus the neutral area (p= 0.0001), and more time was spent with the non estrous female over the neutral area (p=0.03).

4.5.1.2 Analysis of Sexual Behaviour:

Figure 21 represents the percentage of animals displaying sexual behaviour as well as their performance on the three tests. As can be seen from the histogram, not all of the TFMs displayed the full complement of sexual behaviour; however, all of the TFMs displayed, at the very least, mounting behaviour (with pelvic thrusting) on any given mating test. In addition, as many as 50% of the TFMs displayed intromission-like behaviour, with some 30% displaying ejaculation like behaviour. In contrast, on any given test, all of the WT males displayed mounts, intromissions, and ejaculations. An analysis of the latency to mount revealed that the TFMs and WT males exhibited comparable latencies on all three test days. While the TFMs displayed more mounts when compared to the WT males overall, this difference achieved statistical significance only on test day one (T_{18}=2.879, p=0.017). The latency to intromission was shorter in the WT males on days two (T_{18}=2.46, p=0.025) and three (T_{18}=4.18, p=0.002) compared to TFMs. In addition, WT males displayed on average, more intromissions than TFMs, but this was only statistically significant on day two (T_{18}=2.855, p=0.011). Regarding ejaculation latencies, WT males achieved an ejaculation faster than the TFMs on day two,

only ($T_{18}=3.448$, p<0.001); the WT males also displayed more ejaculations when compared to the TFMs on days one ($T_{18}=2.3$, p=0.044) and two ($T_{18}=5.58$, p<0.001). The data are interpreted as suggesting sexual arousal was intact in the TFMs (as evidenced by comparable mount latencies), but because not all of animals in this group displayed the full complement of sexual behavior, the TFMs displayed deficits in sexual performance.

4.5.1.3   Experiment 1b: Sexually Experienced

Following three pairings with a sexually receptive female, both WT males and TFMs were subjected to two more partner preference tests; statistical analysis was run on the mean data of these two tests. The time spent in the estrous chamber by the TFMs was comparable to the WT males ($T_{18}=0.767$; p=0.453). Additionally, TFMs and WT males did not differ in the amount of time spent in the non estrous chamber ($T_{18}=0.653$; p=0.523) or time spent in the neutral zone ($T_{18}=1.341$; p=0.197) (see figure 22).

WT males: For the WT males, an ANOVA revealed an overall significant effect ($F_{2, 24}=19.833$, p<0.001) and the *post hoc* analysis revealed that the WT males preferred to spend more time in the chamber with the estrous female compared to the non-estrous female (p<0.0001), and when compared to the time spent in the neutral area (p<0.0001) (see figure 23). The amount of time allocated to the non-estrous and neutral areas was also different (p=0.002).

TFM affected males: The average time in seconds (+/- SEM) spent in each chamber is represented in Figure 24. For the TFMs, the ANOVA revealed an overall difference in the amount of time spent in each chamber ($F_{2, 27}=10.364$, p=0.00046), and the *post hoc* analysis revealed that the TFMs chose to spend more time with the estrous

female compared to time spent with the non-estrous female (p=0.0002), or in the neutral area (p=0.002). The time spent in the chamber with the non estrous female did not differ from the mean time spent in the neutral area (p=0.441).

4.5.2 Partner Preference Experiment 2: Male and estrous female as stimulus animals

Figure 25 displays the average (+/-SEM) time spent with either the WT male or estrous female. The overall ANOVA revealed a significant between subjects effect for time spent in the estrous female chamber ($F_{2, 17}$=5.645, p=0.013), time spent in the male chamber ($F_{2, 17}$=5.0006, p=0.02), but no significant overall effect of time spent in the neutral area ($F_{2, 17}$=2.6, p=0.103). The *post hoc* analysis revealed WT males and TFMs did not differ in the amount of time spent in the estrous female chamber (p=0.718), however, both differed from the female (compared to male: p=0.018; to TFM: p=0.006). The pattern of results was similar for the non estrous females (male vs TFM: p=0.421; male vs estrous female: p=0.048; TFM vs estrous female: p=0.007). The data suggest the WT males and TFMs acted in a similar manner, which were both different from the estrous female.

WT males: In terms of the partner preference analysis, the ANOVA revealed a significant difference in the amount of time spent in each of the chambers for the WT male ($F_{2, 15}$=15.823, p=0.0002). In this paradigm, the WT males chose to spend more time with the estrous female over the male (p=0.001), and spent more time the with the estrous female than in the neutral area (p<0.0001). However, time spent with the male did not differ compared to time spent in the neutral area (p=0.145) (see figure 26).

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TFM affected males: For the TFMs, the overall ANOVA revealed a significant difference in the average time spent in each of the chambers ($F_{2, 18}=11.337$, $p=0.001$). The TFMs spent the more time in the estrous female's chamber compared to both WT males chamber ($p=0.001$) and the neutral area ($p=0.00045$). The time spent in the neutral chamber was not different than time spent in the WT male chamber ($p=0.754$) (see figure 27).

Estrous females: For the estrous females, the overall ANOVA revealed a significant difference in the average time spent in each of the chambers ($F_{2, 18}=5.433$, $p=0.014$). *Post hoc* analysis revealed this group spent more time with the male than with the estrous female ($p=0.019$). Females also spent more time with the male than in the neutral area ($p=0.007$), however, the time spent with the estrous female did not differ from the time spent in the neutral area ($p=0.633$) (see figure 28).

4.6   Discussion

In the current report, partner preference of TFMs was analyzed using two different paradigms; TFMs were presented with a choice between an estrous and non estrous female in one version of the partner preference (*Experiment 1 a* and *b*), in another version, TFMs, WT males, and estrous females were given a choice between a sexually active WT male or an estrous female. Both WT males and TFMs preferred to spend more time with an estrous female over a male or a non estrous female; however, females preferred a male over an estrous female.   In terms of mount latency, an index of sexual arousal, the TFMs did not differ from WT males, suggesting comparable sexual motivation.   Although they display all three patterns of masculine sexual behaviour, TFMs displayed reduced intromission-like and ejaculation-like behaviour.   Thus, in

agreement with previous reports (Beach and Buehler, 1977), TFMs displayed sexual performance deficits suggesting the androgen receptor mutation specifically affects the ability of these animals to display intromissions and ejaculations.

Partner preference has been examined in androgen insensitive mice (Bodo and Rissman, 2007). In this study, affected males did not choose to spend more time with an estrous female over a wild type male, and this behaviour was similar to that of the experimental female group. However, wild type males spent more time in the area containing the estrous female. Thus, it appears that partner preference is not masculinized in the TFM mouse. The authors suggest that perhaps a failure in masculinization of the vomeronasal system played a role in a lack of partner preference in the TFMs, as bedding from a male stimulated Fos immunoreactivity in regions of the brain important for sexual behaviour (i.e., the hypothalamus) in a similar manner to the females, but failed to stimulate Fos at all in the hypothalamus of the wild type males. However, the distribution of Fos immunoreactivity was not assessed following exposure to estrous female bedding. One important difference between that previous study and the current one concerns the testing apparatus. Jackets were used to tether our 'bait' animals to the back of each of the lateral chambers; this allowed the experimental animals the ability to directly interact physically with the bait animals. In the Bodo and Rissman (2007) study, however, the bait animals were behind wire mesh screens, and thus direct physical contact was prohibited. Stern (1970) showed that sexually naïve male rats did not make a preference for the odor of an estrous female, this was only established when these animals were allowed to mount. TFMs used for the Bodo and Rissman (2007) study were sexually naïve for the partner preference tests, and thus had never mounted an

estrous female before tests in the partner preference apparatus. In the current report, mounting behaviour was observed to be displayed by some of the TFMs and WT males in the partner preference test in which animals were presented with either an estrous or non estrous female. Thus, the inability to physically interact with the experimental animals may have played a role in the lack of a partner preference in the Bodo and Rissman (2007) experiment. However, one could also argue that the differences in the mutation of the androgen receptor between mice and rats result in differences in androgen sensitivity and this contributed to the disparity in results.

The TFM mutation in the rat is a base pair substitution in the ligand binding domain of the translated protein (Yarborourh, et al., 1991). The mutation in the TFM mouse is a frame shift mutation in the amino terminus, resulting in a frame shift in which there is an abberant coding of a premature stop codon; as such, a truncated protein is produced (He, et al., 1991). In order to support the claim that the TFM rat and mouse differ in terms of sensitivity, it becomes crucial, then, to determine the ability of each of the mutated androgen receptors to bind their cognate ligand (i.e., androgens such as testosterone or dihydrotestosterone), dimerize with other bound androgen receptors, migrate to the nucleus, and finally, interact with the chromatin to ultimately up or down regulate protein production. In the TFM mouse, a previous report indicated that the mutated androgen receptor displayed the ability to bind DNA (as assayed via DNA-cellulose chromatography) (Wieland, Fox, Savakis, 1978). Additionally, Schenkein, Levy, Bueker, Wilson (1974) reported induction of protease A activity, albeit, at a much lower rate than in the wild type tissue, in the TFM mouse submandibular gland following treatment with testosterone propionate. This effect appears to be mediated via the

androgen receptor as 17beta estradiol was ineffective, but DHT induced protease A production (Maruyama, Hosoi, Ueha, Tajima, Sato, Gresik, 1993). Additionally, androgen receptors are not 100% absent in the brain of the TFMs, on the contrary, reduced levels of mutated androgen receptors have been detected in the hypothalamus (Wieland, Fox, Savakis, 1978) and cerebellum (Fox, 1977) and bind androgens such as DHT with similar affinity as the wild type protein (Gehring and Tomkins, 1974). Reduced amounts of available mutated androgen receptor, but wild type like affinity for androgens by this receptor, have also been reported in the TFM rat (Roselli et al., 1989). However, there have been no convincing demonstrations that this mutated androgen receptor has any significant physiological activity in both the TFM rat and mouse. Developmentally, the presence of this mutated androgen receptor is unable to induce the masculine phenotype or rescue cells in the spinal nucleus of the bulbocavernosus during ontogenic cell death (Breedlove and Arnold, 1980), does not fully masculinize cells in the medial preoptic area, suprachiasmatic nucleus, or the medial amygdala (Morris et al., 2005), and cannot reduce the high endogenous serum luteinizing or follicle stimulating hormone levels observed in TFMs (Naess et al, 1976). In all these systems, the TFM behaves as though it is completely androgen insensitive. Evidence that is often cited suggesting residual activity in the TFM rat is the Naess et al., (1976) study in which testosterone was injected into TFMs and a noted reduction in LH and FSH activity was observed. However, the amount of testosterone injected was well beyond the physiological normal range, and thus, it is possible secondary or non specific binding to other receptor types produced the observed effects.

Previous reports have examined TFM sexual behaviour and our data seem to agree that the mutation results in reduced levels, but not complete abolition, of intromission-like and ejaculation-like behaviours (Beach and Buehler, 1977). However, data regarding pre-copulatory/appetitive behaviour from TFM rats, such as latency to mount, have not previously been reported. Mount latencies have been considered another measure reflecting the motivation to engage in sexual behaviour where animals displaying short latencies are considered to be highly sexually aroused. In our lab, it has been consistently observed that TFMs display mount latencies in the male range, as well as more mounts with pelvic thrusts but only on the first day of sexual behaviour testing (current report and unpublished observations). One could argue that the ability to transition from mounts to intromissions and ejaculations requires stimulation from the penis, and the sexual performance deficits displayed by the TFMs is solely due to the fact they have a feminine external phenotype. However, if this were the case, why do some TFM rats and mice consistently display the intromission and ejaculatory pattern (current report; Beach and Buehler, 1977; Olsen, 1979 and personal communication)? Given that TFMs displayed a masculinized partner preference as well as masculine mount latencies, it appears that the androgen receptor does not play an important role in the organization of this aspect of pre-copulatory sexual behaviour. Regarding sexual preferences, androgen receptor activation is not necessary for the organization or activation of partner preference; support for this claim comes from a study in which prenatal inhibition of androgen receptors, via injections of flutamide, did not affect the development of masculine partner preference (Dominguez-Salazar et al., 2002). However, studies have shown that androgens, acting upon the androgen receptor, can mediate the activation of

partner preference in adulthood. For example, blockade of the androgen receptor, via administration of hydroxyflutamide, resulted in a decrease in the preference for an estrous female (Vagell and McGinnis, 1998). Additionally, blockade of androgen receptor containing cells within the posterodorsal medial preoptic nucleus (McGinnis et al., 2002) or the ventral medial nucleus (Harding and McGinnis, 2004) inhibited the restoration of a masculinized partner preference. Thus, our data seem to be at odds with these previous studies.

Because androgens can be converted to estrogens, such as 17 beta estadiol (E2) via the enzyme, aromatase, several studies have attempted to link estrogen production and receptor activation to the masculinization of partner preference. Indeed, TFMs have large amounts of available estrogen (Roselli, et al., 1987), binding of estrogen appears to be normal in the brains of these animals (Olsen and Whalen, 1982), and thus it seems likely that perhaps testosterone, after metabolism to E2, could be regulating the masculine partner preference observed in the current report. Prenatal and neonatal administration of the aromatase inhibitor, 1,4,6-androstatriene-3,17-dione (ATD), to rats resulted in a decrease in the preference for an estrous female (Brand et al., 1991; Bakker et al., 1993; Bakker et al., 1995). One study also showed that systemic ATD treatment reduced intromissions and severely impaired ejaculatory responses, but had no effect upon mounting behaviour when animals were treated in adulthood (Bakker et al., 1993). In addition, neither male aromatase knockout mice (Bakker et al., 2002) nor estrogen receptor knockout mice (Wersinger and Rissman, 2002) displayed a masculinized partner preference for an estrous female. However, evidence indicates that ATD competitively inhibits testosterone's ability to transactivate the androgen receptor (Kapplan and

McGinnis, 1989), suggesting ATD has more than one physiological effect. Finally, systemic inhibition of aromatase, via infusions of fadrazole (a more specific aromatase inhibitor), did not have an effect on the ability of adult male rats to display a masculinized partner preference (Vagell and McGinnis, 1997). Thus, it appears that estrogen production and receptor activation may not play a major role in activating partner preference.

4.7   Summary and Conclusions

The current study further refines our understanding of the deficit in mating displayed by the TFM rat. That is, the mutation does not seem to affect the ability of these animals to display masculinized partner preferences and male like latencies to begin mounting. The androgen receptor, thus, seems to specifically affect the performance aspects of sexual behaviour, namely, intromissions and ejaculations.

Figure 17 Schematic of partner preference apparatus.

50.0 in.

24.5in.

13.0in.
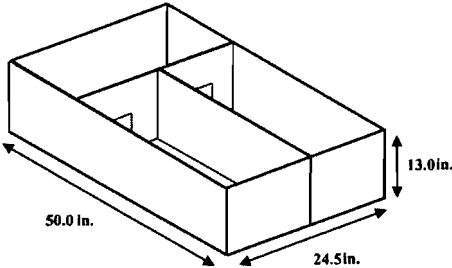
Figure 18 Partner preference in WT males and TFMs in Experiment 1a (sexually naïve). WT males and TFMs did not differ in their performance in the partner preference apparatus (see text for details).

Partner Preference (Sexually Naive)
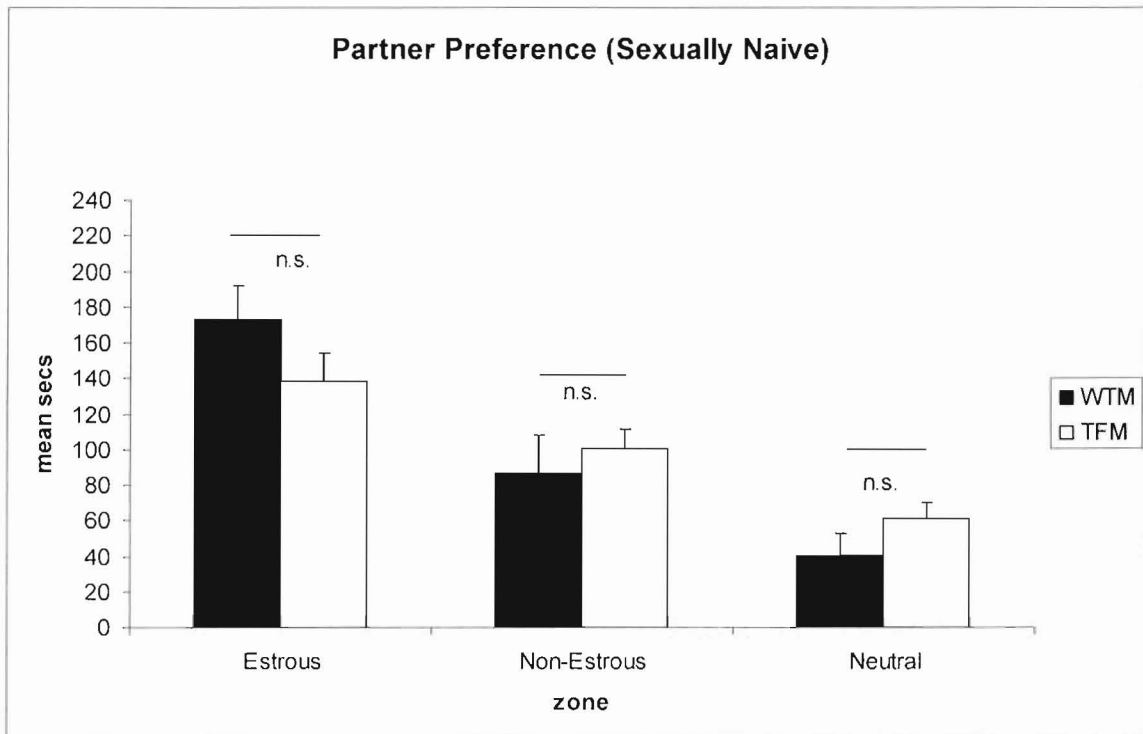
Figure 19 Partner preference performance in WT males. WT males spent more time with the estrous female compared to time spent with the non estrous female and in the neutral zone (see text for details). Note: *= significant comparison; n.s.= non significant comparison

Partner Preference in WT Males (Experiment 1a)
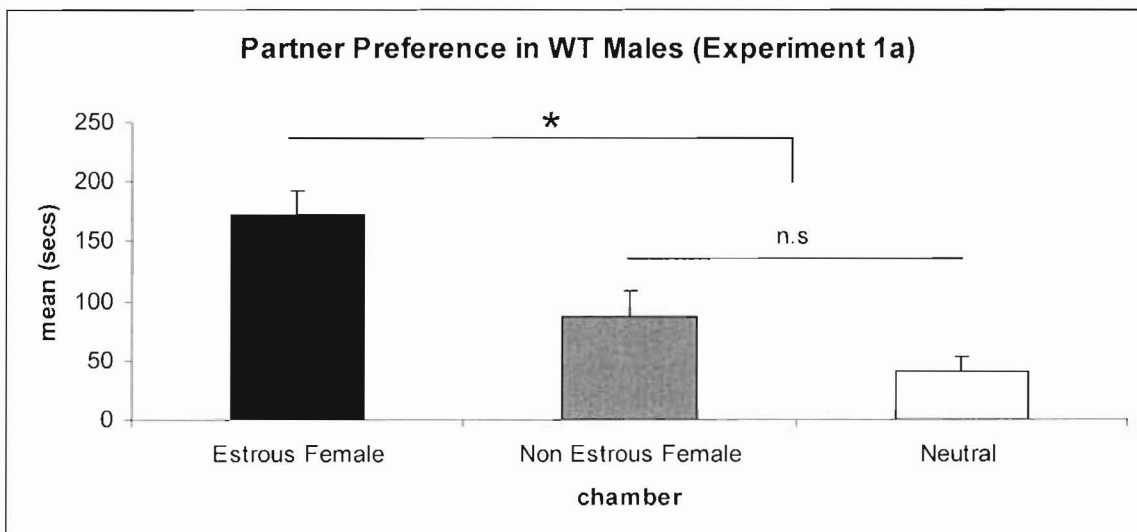
Figure 20 Partner preference performance in TFMs. TFMs preferred to spend more time with the estrous female compared to the non estrous female and neutral zone (see text for detail). Note: *= significant comparison.
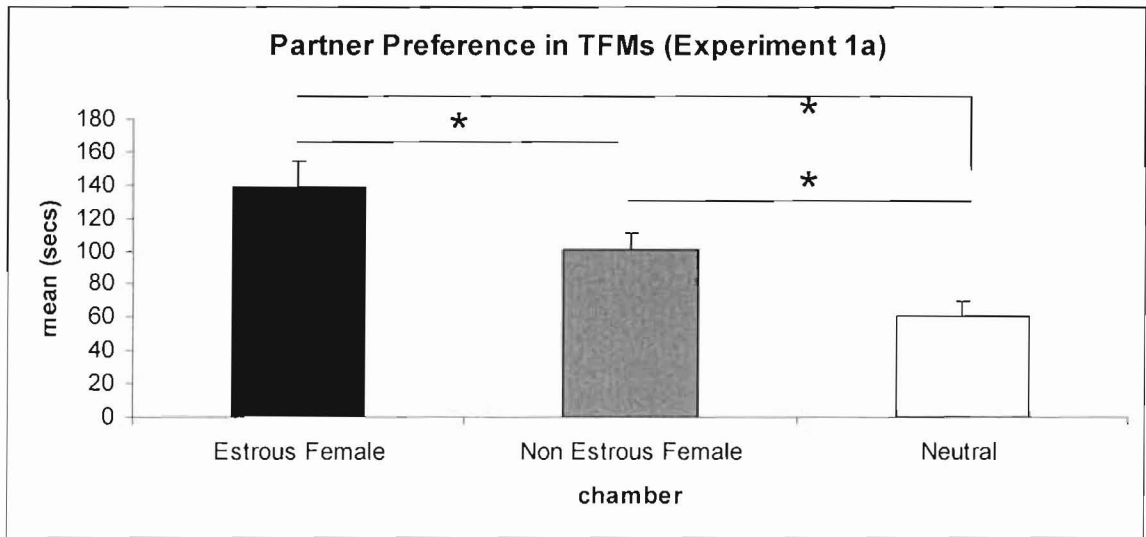
Figure 21 Sexual behaviour performance in WT males and TFMs. Note: *= significant comparison (see text for detail).
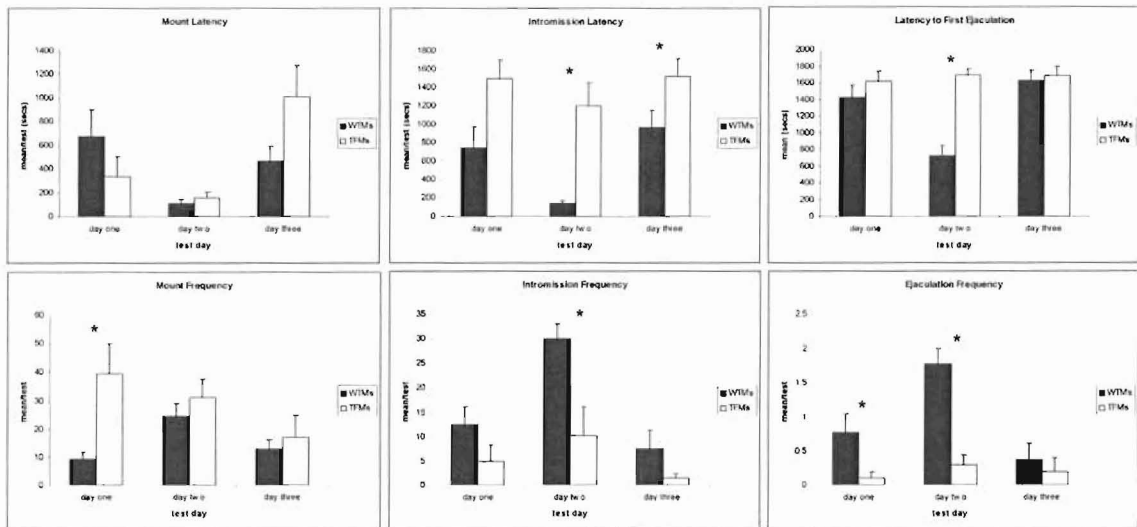
Figure 22 Figure 6. Partner preference performance in WT males and TFMs in Experiment 1b
(sexually experienced). Performance between WT males and TFMs did not differ.
Note: *=significant comparison; n.s.= non significant comparison (see text for details).
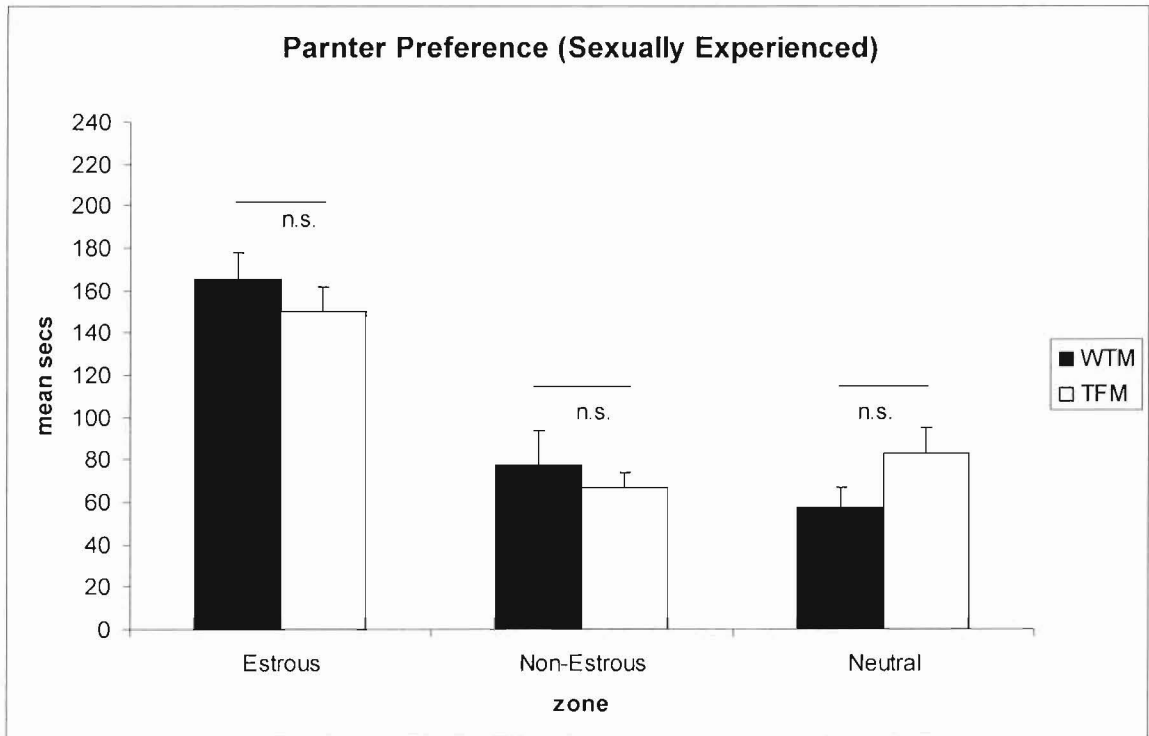
Figure 23 Figure 7. Partner preference performance in WT males. WT males preferred to spend
time with the estrous female over the non estrous female and neutral zone. Note: *=
significant comparison (see text for details).
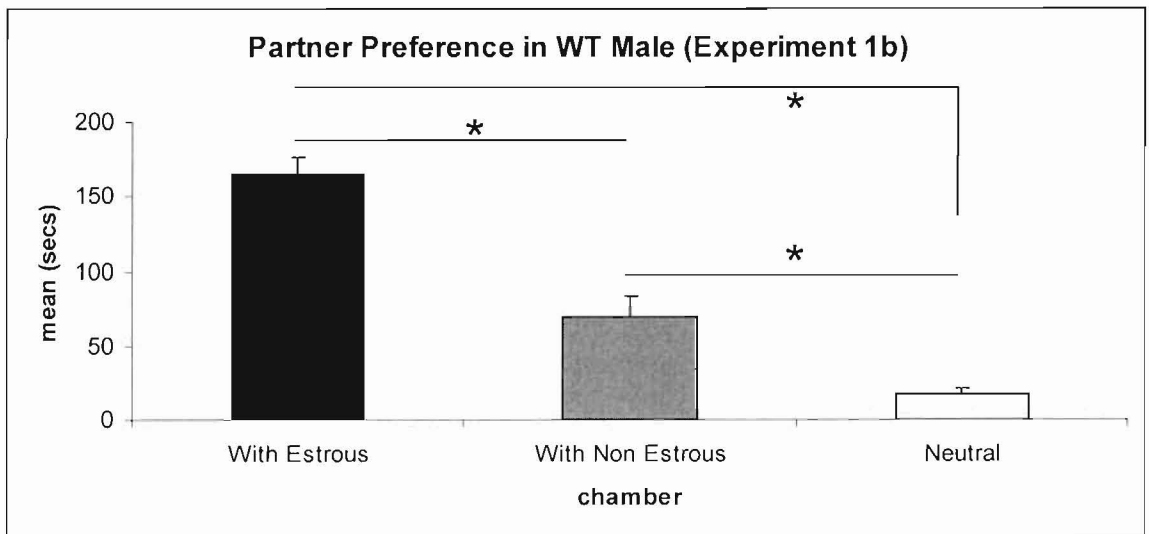
Figure 24 Partner preference performance in TFMs. TFMs preferred to spent more time with the estrous female over the non estrous female or neutral zone. Note: *=significant comparison; n.s.= non significant comparison (see text for details).
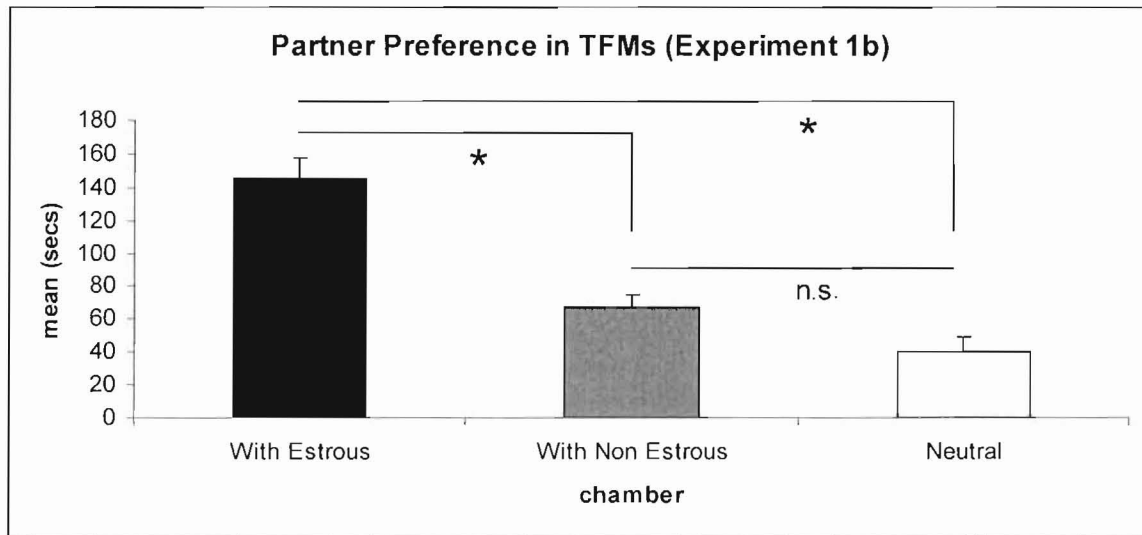
Figure 25 Partner preference in WT males, TFMs, and estrous females in Experiment 2. Both WT males and TFMs performed similarly, whereas both were different from the estrous female. Note:*= significant comparison; n.s.=non significant comparison (see text for details).
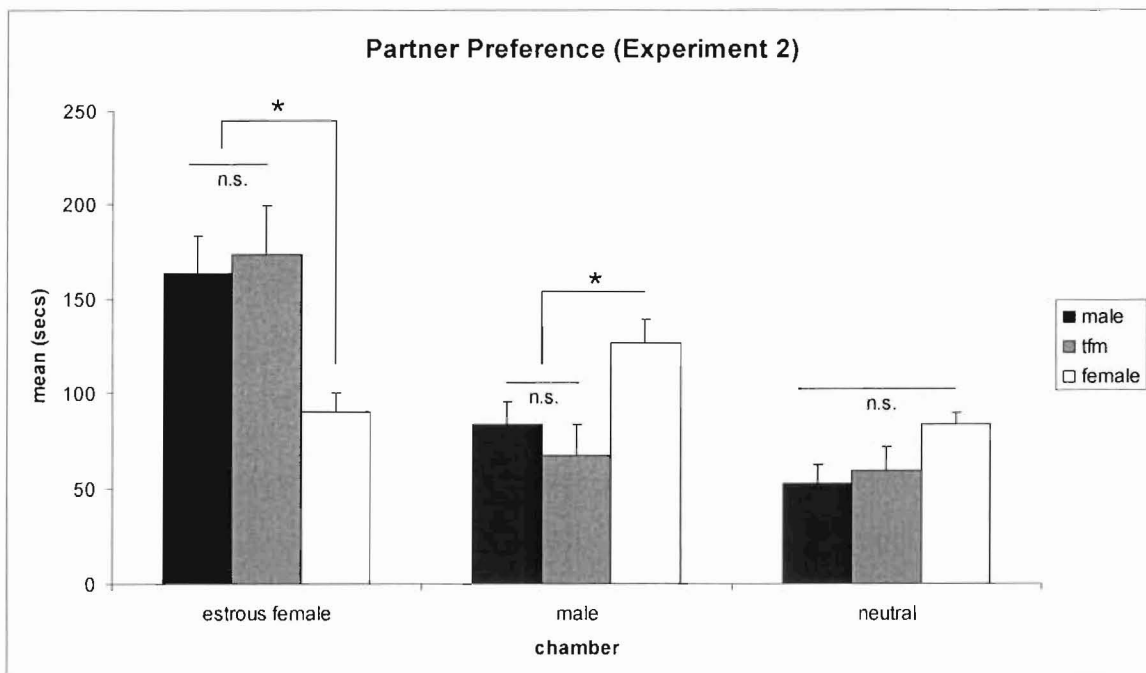
Figure 26 Partner preference performance in WT males in Experiment 2. WT males preferred to spend the most time with the estrous female over the male or neutral zone. Note: *=significant comparison.
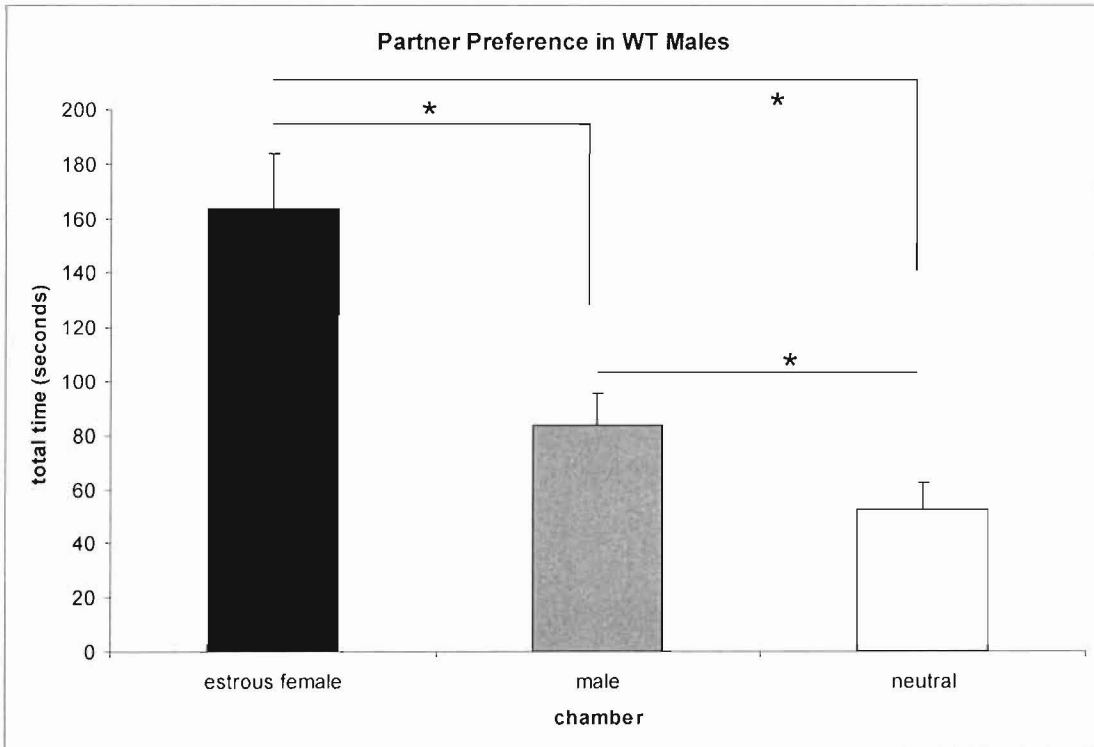
Figure 27 Partner preference performance in TFMs. TFMs preferred to spend time with the estrous female over the male or neutral zone. Note: *=significant comparison; n.s.=non significant comparison.
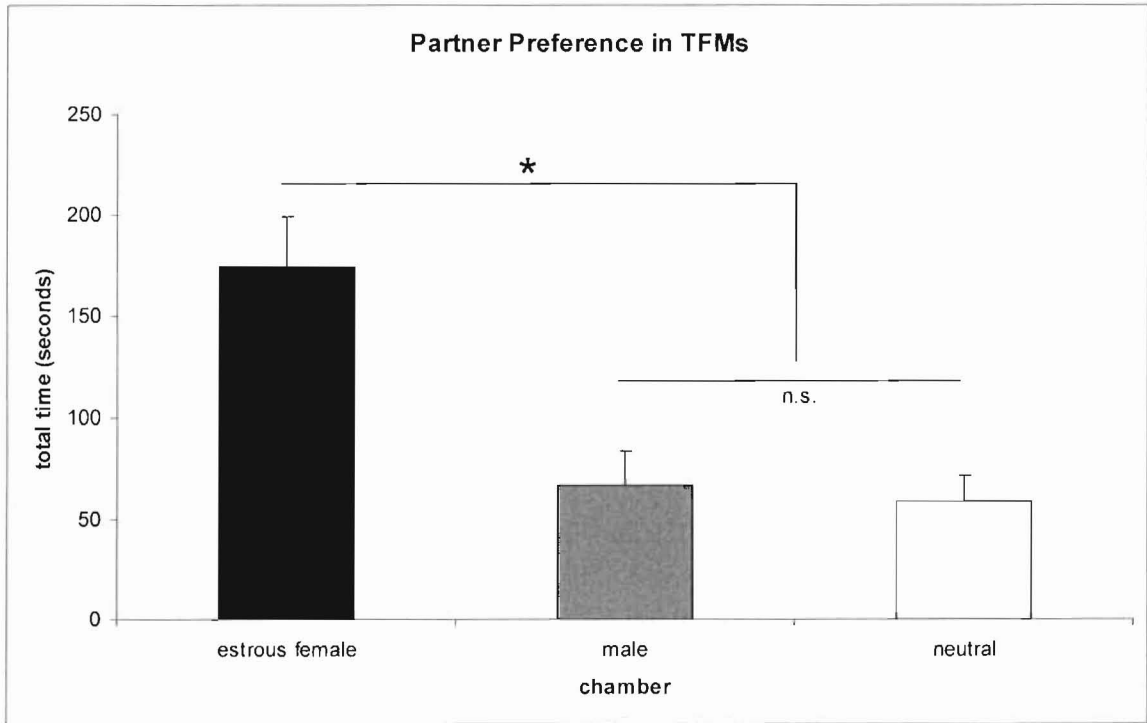
Figure 28 Partner preference performance in Estrous females. Estrous females preferred to spend time with the sexually active male versus the non estrous female or the neutral chamber. Note: *=significant comparison; n.s=non significant comparison.
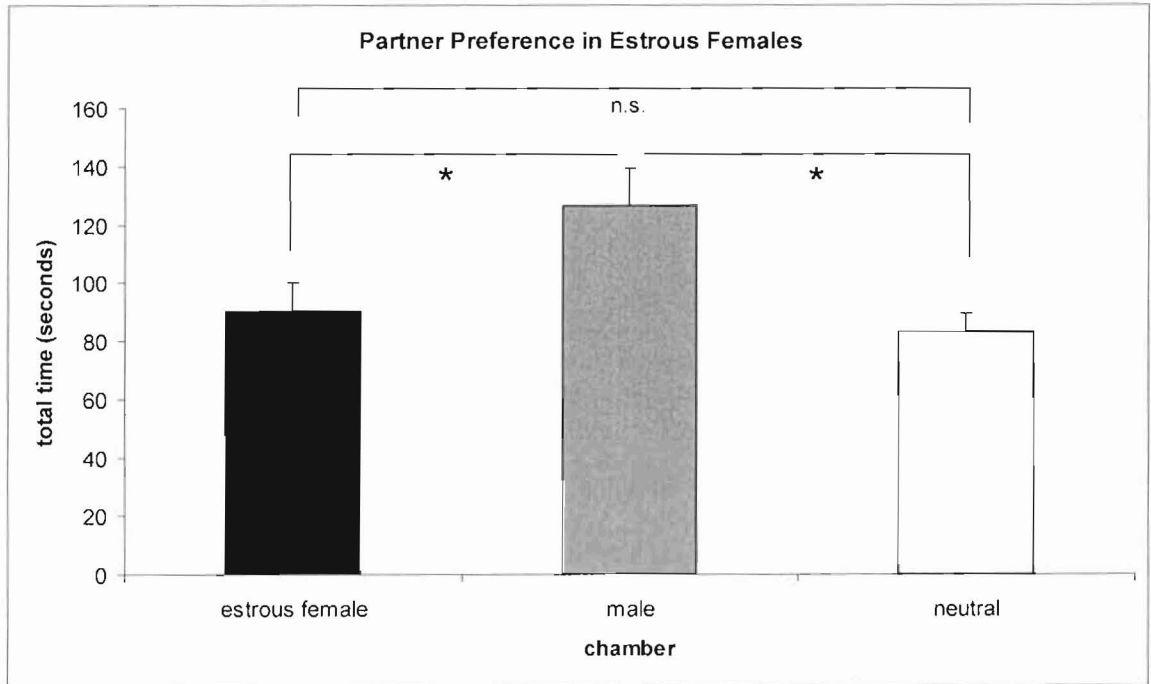
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CHAPTER 5 FOS IMMUNOREACTIVITY AND MORPHOMETRIC ANALYSIS OF THE ACCESSORY OLFACTORY BULB IN MALE RATS CARRYING THE TESTICULAR FEMINIZATION MUTATION

5.1 Abstract:

In the current report, we examined the morphometry and distribution of Fos in the

accessory olfactory bulb (AOB) of male rats carrying the testicular feminization mutation

(TFMs). Previous reports have indicated that TFMs display sexual performance deficits.

As a crucial participant in the detection of pheromones, the AOB is thought to contribute

to precopulatory behaviours such as ultrasonic vocalizations (USVs) and may stimulate

mounting behaviours, important for stimulating intromissions and ejaculations. Sexually

inexperienced wild type (WT) and TFM affected males were mated several times with an

estrous female. Following sexual experience, Fos immunoreactivity, a marker of cellular

activation, in the AOB olfactory bulb was compared between WT males and TFMs.

Additionally, we quantified the somatic area of cells in the mitral cell layer and compared

these parameters between the WT males, TFMs, and estrous females. The areas of the

granule and mitral cell layers, as well as the soma of the mitral cells, were fully

masculinized in the TFMs. A ratio of mitral cell layer versus granule cell layer Fos,

which we suggest may be an overall indicator of GABA-ergic activity was higher in WT

males than TFMs, suggesting decreased AOB ouput in the TFMs. Thus, androgen

receptor stimulation may be necessary for the proper functioning of the AOB, however,

the morphometric analysis suggests overall, the AOB is masculinized.

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5.2   Introduction

Rodents have two parallel systems for the detection of environmental odorants. One system, the vomeronasal system (VNS), is comprised of the vomeronasal organ (VNO) and the accessory olfactory bulb (AOB), and the other, the main olfactory system (MOS), is comprised of the nasal epithelium and main olfactory bulb. Projections of the VNO reach the hypothalamus by way of the medial amygdala (Scalia and Winans, 1970), whereas the MOS primarily projects to areas in the forebrain and cortex (Scalia and Winans, 1975; de Olmos, Hardy, Heimer, 1978). Experimental manipulation of the VNS impairs the execution of appetitive and consumatory sexual behaviours (Stowers, Holy, Meister, Dulac, Koentges, 2002), whereas destruction of the MOS largely leaves these displays intact. Studies suggest the VNS encodes information regarding mating status and gender information (Luo, Fee, Katz, 2003), whereas the MOS largely detects other non-reproductive related odorants, however, recent evidence seems to challenge this distinction (Mandiyan, Coats, Shah, 2005).

Male rats carrying the testicular feminization mutation (TFMs) display a feminized external phenotype (blind ending vagina, nipple line, short anogenital distance), but contain abdominal testes that are androgen secretory (Allison, Chan, Stanley, Gumbreck, 1971). This feminine phenotype in the TFMs is due to a mutation in the gene encoding the androgen receptor (Yarborough, Quarmby, Simenta, Joseph, Sar, Lubahan, Olsen, French, Wilson, 1990), as receptor activation during a critical period is necessary for the masculinization of the external genitalia. The mutation resides in the steroid binding domain of the translated protein, resulting in an inability of the protein to properly interact with the chromatin, essentially inhibiting its ability to effect

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transcription. Previous studies have shown that these animals fail to display the full complement of masculine sexual behaviour (Shapiro, Goldman, Steinbeck, Neuman, 1975; Beach and Buehler, 1977; Olsen, 1979; Shapiro, Levine, Adler, 1980), and it has been suggested that this is due to a lack of aromatization of testosterone to estrogen in adulthood (Olsen, 1979). Arguing against this, though, are studies showing that serum estrogen levels are higher than wild type (WT) male values (Roselli, Salisbury, Resko, 1987), with the level of estrogen binding in the hypothalamus being comparable between TFMs and WT males (Olsen and Whalen, 1982). Together, the data suggest the estrogen receptor system is intact in the TFMs. Moreover, given that gonadal steroids also have organizational effects and that numerous neural and peripheral structures express androgen receptors, it is possible that a lack of androgen receptor activation during the critical developmental period for masculinization of the structures important for the display of sexual behaviour could also contribute to the behaviour deficits in TFMs.

Given that the AOB functions to relay important information about the estrous status of the female rat, which in turn initiates the display of mounting behaviour, it is possible that mating deficits in TFMs are partly due to an inability to properly respond to estrous pheromones. Additionally, evidence implicates the VNS in the induction of 50 kHz ultrasonic vocalizations (Bean, 1982), an important precopulatory behaviour which reportedly increases sexual receptivity in the estrus female (White and Barfield, 1990; White, Cagiano, Barfield, 1990). We have recently observed significantly reduced production of 50 kHz USVs in the TFMs following exposure of an estrous female (Hamson, Csupity, Gaspar, Watson, submitted). In the current study, we have assessed the volumes of the glomerular, mitral cell, and granule cell layers in the AOB and have

quantified the somatic area of the cells in the mitral layer. WT and TFM affected males were mated with an estrous female several times, and the amount of Fos immunoreactivity (a marker of cellular activation; Tischmeyer and Grimm, 1999) in the mitral and granule cell layers was assayed on the final test of sexual behaviour in order to determine if there were any abnormalities in AOB cellular activation in the TFMs.

5.3   Methods

5.3.1   Animals

Animals for this study were obtained from our colony at Simon Fraser University. Female rats (n=4) previously identified as being heterozygous for the testicular feminization mutation (i.e., carriers) were bred with stud males obtained from the University of British Columbia (Vancouver, Canada). Briefly, carriers and stud males were housed for several days in cages containing a wire mesh bottom allowing for a vaginal plug to fall through the bottom onto newspaper covered trays; the presence of a vaginal plug indicated mating had taken place. At this time, carriers and stud males were separated and placed back in their home cages for gestation. Three weeks following parturition, litters were separated based on phenotypic markers; males were identified as having a long anogenital distance and a scrotal sac. Male rats carrying the testicular feminization mutation (TFM), as well as wild type and carrier females (termed unidentified daughters of carriers; UDOCs), were identified as having a short anogenital distance and no scrotal sac. Because TFMs and UDOCs are indistinguishable based on phenotypic markers, TFMs were further identified using genetic markers amplified using a polymerase chain reaction and subjected to a restriction endonuclease (details in Fernandez et al., 2003) (final animal numbers are presented below).

5.3.2   Fos Immunocytochemistry

Sexually experienced WT males and TFMs were paired individually with an estrous female for 30 minutes and allowed to freely copulate. One hour after the thirty minute test, WT males (n=8), TFMs (n=8), and estrous females (n=6) were killed using a blended mixture of CO_2 and oxygen. The animals were transcardially perfused with approximately 60 ml's of 0.1M PBS, followed by approximately 60 ml's of 4% Paraformaldehyde diluted in PBS. The olfactory bulbs were harvested and then immediately placed in 4% paraformaldehyde diluted in 0.1M PBS for two hours. Tissue was then cryoprotected in 30% sucrose dissolved in 0.1M PBS overnight. The olfactory bulbs were mounted on a frozen stage with M1 embedding medium (Lipshaw, Pittsburgh, PA) and sectioned in the sagittal plane at 50μm intervals using a sledge microtome (American Optical Company; model 860), into three series of sequential sections. Sections were stored in an antigen sparing solution (DeOlmos; Watson et al., 1986), and placed in the freezer (-20 °C) until processed for Fos immunoreactivity (Fos-ir) or stained for Thionin. One series consisting of every third section was processed for Fos immunoreactivity, whereas another series was stained with thionin.

Accessory olfactory bulbs were processed for Fos-ir using the free floating method carried out in custom made tissue wells. All sections were washed with PBS containing triton X-100 (Sigma Chemicals, St. Louis, MO) for 3x5 minutes at room temperature in between each of the reactions listed below. Sections were blocked in 10% normal goat serum (Vector Laboratories, Burlington, ON), followed by an overnight incubation at 4°C with a Fos polyclonal antibody raised in goats (1:2000; sc52G; Santa Cruz Biotechnology, Santa Cruz, CA). The tissue was then incubated in a goat anti rabbit secondary (1:200; Vector Laboratories, Burlington, ON) for one hour. Following this,

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the tissue was incubated in an avidin-biotin horseradish peroxidase complex according to the manufacture's instructions (Vector Laboratories, Burlington, ON). Antigen sites were visualized using 3,3'-diaminobenzidine diluted in 0.1M Tris buffer (pH 7.2), containing hydrogen peroxide (60ul of 30% H_2O_2) and 8% nickel chloride (100 ul). Sections were mounted on gelatin coated slides, dehydrated in a graded series of ethanol, cleared in xylene, and then coverslipped with Permount (Sigma Chemicals, Oakville, ON).

In order to analyze soma size in the mitral cell layer (MCL) and the total area occupied by the glomerular, mitral cell , and granule cell layers, another series of olfactory bulb sections was mounted on gelatin coated slides and allowed to dry overnight on the bench top. The next day, slides were rehydrated in deionized water, stained with thionin, differentiated with 70% ethanol containing acetic acid, dehydrated in a graded series of ethanol solutions, cleared in Xylene, and then cover slipped with Permount.

5.3.3   Image Analysis

5.3.3.1   Quantification of Fos immunoreactivity (Fos-ir)

Regions of interest (ROIs) (magnified using a 20x objective; final magnification= 200x) containing the accessory olfactory bulb (AOB) were captured using a 3ccd camera (Sony Canada) connected to a microscope (Nikon Eclipse E600; Nikon Canada, Mississauga, ON) by a researcher blind to experimental conditions. Digitized images were imported to a computer for analysis using AIS-C/MCID software (Imaging Research Inc, St. Catherines, ON). Cells containing Fos-ir were counted in the mitral and granule cell layers with the aid of an automated grain counting routine. First, the imported images

were converted to grey scale (256 levels of gray). Then, valid targets (i.e., cells containing Fos-ir) were dinstinguished from background via the use of a thresholding adjustment that established a minimum optical density cutoff. Pixels lying beneath the density cut off range were deemed as representing non specific background staining (and thus ignored), whereas pixels with a density above the established cut off range were deemed as valid targets (i.e., Fos-ir cells) by the AIS software. Once the criteria for a valid target was set, a sampling tool (110μm x 110 μm) was used to quantify the number of Fos-ir cells, collected from a minimum of four sections per animal. For each mitral and granule cell layer analyzed, the sampling tool was randomly placed over top of the granule cell layer and the number of valid targets was automatically quantified; counts were adjusted manually if deemed necessary.

5.3.3.2   Morphometry of the Accessory Olfactory Bulb (AOB)

The overall areas of the glomerular, mitral cell, and granule cell layers were quantified from Nissl stained sagittal sections. These regions were magnified using a 10x objective (final magnification= 100x), and the pictures were again imported into the AIS-C/MCID software for analysis. A researcher blind to experimental conditions used a tracing tool to outline the areas in the AOB, and the software then automatically computed an area (in μm^2). For soma sizes, cells in the mitral layer were magnified using a 60x objective (final magnification= 600x), and 40 cells, chosen at random, were outlined using a tracing tool; the software automatically computed an area (in μm^2) for each cell. In order for the cells to be included in the analysis, a clear nucleus and nucleolus had to be visible. Under the microscope, these cells were very large, compared to presumptive glia, and thus we were confident that they neurons.

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5.4 Statistical Analyses

Possible group differences in Fos immunoreactivity were detected using two-tailed independent samples T tests, with $\alpha=0.05$ set as the rejection criteria. A one way analysis of variance (ANOVA; SPSS 14.0) was used to detect possible differences in area and soma size between the different genotypes; overall alpha set at $\alpha=0.05$. Where appropriate, *post hoc* comparisons were made using Fisher's least significant difference statistic.

5.5 Results

5.5.1 Fos Immunoreactivity in the Accessory Olfactory Bulbs

A schematic diagram of the accessory olfactory bulbs (and the vomeronasal organ) is presented in Figure 29. Figure 30A displays the mean (+/- SEM) number of Fos-ir cells in the mitral and granule cell layers of the WT males and TFMs. Descriptively, the average number (+/-SEM) of Fos immunoreactive (Fos-ir) cells in the mitral layer for the males was (9.56 +/-1.1) compared to the TFMs (7.99 +/-1.1), whereas the TFMs (18.6 +/-2.1) displayed, on average, more Fos-ir cells in the granule cell layer compared to WT males (14.1 +/-2.6). We chose to represent the data as a ratio of activated cells in the mitral layer to activated cells in the granule cell layer given that they act as a unit and gives a better representation of the functional output of the AOB as a whole (see discussion for details); This ratio is represented in figure 30B. WT males displayed a higher proportion of activated mitral cells to granule cells (82.16% +/-7.6) compared to the TFM affected males (44.87% +/-5.8) as revealed by a two-tailed T test of mean differences ($T_{10}=3.767$, $p=0.004$). Figure 31 contains representative photomicrographs of

Fos immunoreactivity on thionin stained sections from both WT and TFM affected

males.

5.5.2 Morphometry of the Accessory Olfactory Bulbs

5.5.2.1 Area Analysis

Figure 32 contains the group means in regional volume of the glomerular, mitral, granule

cell layers. Figure 33 contains representative photomicrographs of these layers in the WT

male, female, and TFM. The ANOVA did not reveal an overall affect of genotype on the

area of the glomerular layer ($F_{2,21}$=3.237, p=0.06). However, the ANOVA revealed a

significant effect of genotype on the mitral cell layer ($F_{2,21}$=8.922, p=0.002), and the

granule cell layer ($F_{2,21}$=15.116, p<0.0001). The *post hoc* analysis revealed a sex

difference for the granule cell layer, as the WT males displayed a larger cross sectional

area compared to the WT females (p=0.001). The *post hoc* analysis of the mitral cell

layer also revealed a sex difference, as the WT males again displayed a larger area than

the females (p=0.00003). Compared to the TFM affected males, WT females also

displayed smaller cross sectional areas in both the mitral (p=0.009) and granule cell

layers (p=0.0002), however, TFM affected and WT males did not differ on these

parameters. Thus, we can conclude from the data that the glomerular layer does not

display a sex difference, however, both the mitral and granule cell layers do.

Additionally, the mitral and granule cell layers are masculinized in the TFM affected

male rat.

5.5.2.2   Soma Size Analysis

For the soma size analysis, only a subset of the WT males (n=5) and females (n=6) was analyzed (all TFMs were analyzed). The ANOVA revealed an overall significant effect of genotype on mitral cell size ($F_{2, 18}$=12.743, p<0.001). The cross sectional area of these cells displayed a sex difference with WT males containing larger cross sectional areas than WT females (p=0.007). TFM affected males also displayed larger somata in the mitral cell layer compared to WT females (p<0.0001), and, WT and TFM affected males did not differ in soma size (p=0.185) (see Figure 32, bottom). We conclude from this that mitral layer cells are sexually dimorphic, and that the size of cells in this layer is masculinized in the TFMs.

5.6   Discussion

The accessory olfactory bulb (AOB) is linked to the vomeronasal organ, located in the nasal septum, and appears to be important for determining the hormonal and receptive status of female rats, which in turn is important for the proper display of masculine sexual behaviour. Previous studies have documented performance deficits in TFM affected male rats and mice, and thus the current report was undertaken to determine if there were any morphological or activational abnormalities associated with the AOB in these mutants. We observed that the TFMs have masculine soma sizes in the mitral cell layer; similarly, their mitral and granule cell layers are masculine in overall size. Finally, the ratio of activated cells (as determined by Fos expression) in the mitral layer to those in the granule cell layer, is higher in the WT males compared to the TFM affected males, suggesting a possible difference in the output of the mitral/granule layer unit.

The accessory olfactory bulb (AOB) is sexually dimorphic in that the area of the AOB and its constituent components, the glomerular, granule, and mitral cell layers have been reported to be larger in males compared to females. Additionally, the number and somatic area of cells in the mitral and granule cell layer has been reported to be sexually dimorphic (Caminero, Segovia, Guillamon, 1991; Valencia, Segovia, Guillamon, 1986a, b). We have replicated the sex difference in terms of the cross sectional area of the mitral and granule cell layers, as well as the somatic extent of cells in the mitral layer; however, we did not observe a sex difference in the glomerular layer. One possibility to account for the discrepancy in the previous study with the current one may have to do with the way in which the tissue was prepared for area analysis; the previous report used a rapid Golgi impregnation method (Caminero, Segovia, Guillamon, 1991), whereas cells were stained with thionin in the current report.

We extend the sex difference in the area of the mitral and granule cell layers, and the soma size of the mitral cells, to include the TFM affected male rat. While we cannot distinguish between organizational and activational effects of androgens on these structures in the current report, previous studies suggests gonadal steroids acting during a critical period of development around the time of birth create the sex difference in the AOB.

Gonadectomy of males or injection of androgens into females postnatally reverses the sex differences in the AOB (Roos, Roos, Schaeffer, Aron, 1988; Caminero, Segovia, Guillamon, 1991). In the brain, the conversion of androgens (such as testosterone) to estrogen serves to attenuate ontogenetic cell death in the major projection areas of the AOB (i.e., the medial amygdala and medial preoptic area) through interaction with

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estrogen receptors. A similar process seems to take place in the AOB. Injection of

estradiol to pups which have been orchidectomized on the day of birth results in a

masculinized AOB in terms of the number of mitral cells, however, injection of DHT was

ineffective (Perez-laso, Segovia, Collado, Rodriguez-Zafra, Del Abril, Guillamon, 1997).

In female rats, it has been suggested that androgens, such as dihydrotestosterone, are

reduced to allopregnanalone or tetrahydrodeoxycorticosterone, which act upon GABAa

receptors to induce cell death in this region during the second week of life (Valencia,

Collado, Cales, Segovia, Perez Laso, Rodriguez Zafra, Guillamon, 1992). Support for

this comes from a report demonstrating that administration of a GABAa receptor agonist,

diazepam, reduced the number of mitral cells in the AOB of male rats to female levels

(Perez-Laso, Valencia, Rodriguez-Zafra, Cales, Guillamon, Segovia, 1994). Thus, the

data in the current study are consistent with the idea that during the first week of life, the

TFMs were exposed to the masculinizing effects of estrogen acting upon the AOB. No

studies, however, have reported on estrogen content, aromatase activity, or estrogen

receptor numbers in the AOB of developing TFMs.

A previous study reported that the volume of the sexually dimorphic region of the

preoptic area (SDN-POA) in TFMs was also fully masculine as it was also larger than

WT females (Morris, Jordan, Dugger, Breedlove, 2005). In contrast, the volume of the

suprachiasmatic nucleus, as well as the ventrolateral subdivision of ventral medial

hypothalamus (Dugger, Morris, Jordan, Breedlove, 2007), were both reported to be

feminine compared to WT males, while the posterodorsal division of the medial

amygdala exhibited an intermediate volume in the TFMs (Morris, Jordan, Dugger,

Breedlove, 2005). Together, the different studies suggest the androgen receptor mutation

has somewhat selective effects upon different areas of the brain. In the case of the AOB, our data indicate that it is masculinized in the TFM affected male rat.

In general, WT males and TFMs display heavier body weights compared to females, WT males typically have heavier brains than females, and others have observed that the TFMs are masculinized in terms of overall brain weight (Dugger, Morris, Jordan, Breedlove, 2007). Thus, differences observed in the current report in overall brain and body weight may account for the sex difference in the volume of the mitral and granule cell layers. However, Dugger, Morris, Jordan, and Breedlove (2007) argue that differences in overall weight do not appear to predict an overall sexual dimorphism in any one particular brain region; they report that subregions within the ventromedial hypothalamus are dimorphic, whereas others are monomorphic. Additionally, the TFMs displayed feminized subregions within the VMH, again supporting the notion that sex differences are not generalized but specific.

The protein product of the immediate early gene (IEG), *cfos*, is thought to be a marker of cellular activation (Tischmeyer and Grimm, 1999). Previous studies employing Fos as a marker of mating-related activity indicate the medial preoptic area, bed nucleus of the stria terminalis, medial amygdala, and nucleus accumbens are especially activated in male rats allowed to mate to ejaculation (Robertson, Pfaus, Atkinson, Matsumura, Phillips, Fibiger 1991; Baum, Everett, 1992). These regions also generally express androgen and estrogen receptors, forming a steroid sensitive neural circuit, which have been reported to be colocalized with mating induced Fos immunoreactivity (Greco, Edwards, Michael, Clancy, 1998). While certain components of masculine sexual behaviour can stimulate and augment IEG expression in regions

important for the display of mating, the display of Fos in the accessory olfactory bulb appears to be specific to the detection of relevant odorants independent of mounts, intromissions, and ejaculations (Oboh, Paredes, Baum, 1995; Fernandez-Fewell, Meredith, 1994, Matsuoka et al., 2002). Indeed, several studies have observed increased Fos immunoreactivity in the AOB after presentation of heterospecific pheromones, but no increases were seen following presentation of pheromones followed by sexual behaviour.

Reports have shown that VNS stimulation produces a very characteristic pattern of Fos-ir in the AOB; cells in the granule cell layer express more Fos than cells in the mitral cell layer with repeated pairings. This distribution can be explained by the electrochemical properties and the neural organization of this system (Jia, Chen, Shepherd, 1999). Odorants are detected by the vomeronasal organ and this information is sent to the glomerular layer, where axons contact the dendrites of the mitral cells. Activation of the mitral cells causes the release of glutamate, which increases the firing rate of cells in the granule layer. Activation of the granule cells following VNO stimulation reciprocally inhibits the activity of cells in the mitral layer. Increased activation of mitral cells can lead to a greater quantal release of GABA from the granule cell layer, and thus an overall decrease in activity from these cell groups can be expected with repeated activation.

Increased GABAergic tone (i.e., increased inhibitory gain) in the granule cell layer, with subsequent attenuation of mitral cell activity, has been used to explain how the scent of a novel, but not familiar, male may induce pregnancy block (i.e., the Bruce Effect) in female mice (Brennan, Kendrick, Keverne, 1995); the Bruce effect is a phenomenon that is similar to mate recognition. Presentation of females with the scent of

a male does not induce IEG expression in the AOB of unmated females (Brennan, Hancock, Keverne, 1992). However, following copulation, increases in Fos immunoreactivity were observed 1-2 hours later (with maximal stimulation at 3-4 hours) in the granule cell layer. The authors suggest that a reduction of mitral cell activity, due to the pairing of mating with the scent profile of the male, effectively disengages the neuroendocrine reflex that regulates estrous cyclicity. Instead, the luteal phase is extended, leading to a sustained release of progesterone, which prepares the uterus for implantation. Presentation with the scent of a novel male shortly after mating reengages this mechanism possibly through activation of non-strengthened mitral-granule cell synapses, returning the female to behavioural estrous. Inhibition of granule cells via infusions of the allosteric GABAa receptor antagonist, bicuculline, induces pregnancy block when injected following copulation; this may be accomplished through disinhibition of the mitral cells as infusions of bicuculline increase Fos immunoreactivity in this region (Brennan, Hancock, Keverne, 1992). In this way, then, the AOB can be said to act as a comparator of familiar scents to unfamiliar scents, and depending upon the activation pattern of cells in the AOB will determine the activity of the neuroendocrine reflex that regulates estrous cyclicity. Additionally, one can see that the increased GABA-ergic tone would essentially lower the ratio of mitral cell activity to granule cell activity, decreasing the overall output of the AOB.

In the current report, we have observed the TFMs to display a lower ratio of mitral cell to granule cell activity compared to the WT males. The current understanding of AOB circuitry suggests this could be associated with increased GABA-ergic tone, and thus decreased output of the AOB. If activity in these cell populations is important for

mate recognition, then it is possible this pattern of activation could be expected in order to distinguish females in estrous from females not in estrous in male rats. Previous reports indicate that males exposed to estrous females but prevented from physical interaction display non contact erections and increased concentrations of dopamine in the nucleus accumbens, suggesting pheromonal stimulation activated sexual excitement (Fiorino and Phillips, 1999). Subsequent tests with these males resulted in decreased latency to begin mating compared to males that were not previously exposed to an estrous female. What this data suggests is the primed males may have formed an olfactory memory for the estrous female (versus the non primed males), and upon subsequent exposure, displayed shorter latencies to begin mounting.

We have previously observed TFMs to display shorter latencies to mount estrous females compared to WT males, but only on the first day, and have observed an exaggerated mounting response in which TFMs display multiple mounts (with pelvic thrusting) per single elicited lordosis; increased mounting behaviour was also reported by Beach and Buehler (1979). Thus one could speculate that decreased activity from the AOB may lead to increased activation of the circuitry regulating mounting, and in the TFMs, this may account for the exaggerated mounting behaviour. As stated previously, one of the primary projection areas of the mitral layer is the medial amygdala (Scalia and Winans, 1975), a region known to be important for mounting (Dominguez, Riolo, Xu, Hull, 2001). Additionally, it has been reported that increased output of the AOB was associated with a lack of sexual behaviour in male rats that do not display any motivation or sexual behaviour (termed non copulators or 'duds') (Portillo and Paredes, 2004). In this study, the activity (as indexed by Fos-ir) of the GABA expressing cell group in the

granule layer was observed to be 300% lower compared to the males which mated, and

thus the output of the AOB would be expected to be higher than the animals that do

display mating (this ratio was not calculated in this study). In addition, the non

copulators displayed reduced levels of Fos-ir in the medial amygdala (posterodorsal

division) compared to copulating males following presentation with sexually relevant

odorants. We have previously observed increased Fos in the medial amygdala of

sexually experienced TFMs compared to WT males following 7 mounts with pelvic

thrusting (Hamson, Csupity, Ali, Watson, unpublished observations).

There is also another possibility that could explain the pattern of results obtained

in the current report. Regulation of the activity at the dendrodendritic synapse between

the mitral and granule cells is achieved through activation of $\alpha 2$ adrenergic receptors by

noradrenalin released from fibers originating in the locus coeruleus. This receptor

activation leads to decreased intracellular $Ca++$ and a subsequent overall decrease in the

quantal release of glutamate by the mitral cells; the overall effect is an increase in the

firing rate of the granule cells (and thus increased GABA-ergic tone) (Kaba and Huang,

2005). Additionally, it has been reported that the enzyme regulating the production of the

precursor for noradrenalin (i.e., dopamine) contains an androgen response element in the

promoter region of the gene. Thus, given the lack of androgen responsiveness in the

TFMs would suggest an overall decrease in $\alpha 2$ adrenergic receptor activity in the AOB,

essentially increasing the GABA-ergic tone of the area. This possibility negates the need

to explain the pattern of Fos-ir in the mitral and granule cell layers via the formation of

an olfactory memory, and essentially suggests differences in AOB output displayed by

the TFMs are a by-product of how this system is regulated.

It is important to note that we cannot determine the amount of VNS stimulation that each of the groups had in this experiment. Indeed, while a common part of precopulatory sexual behaviour is anogenital investigation, sexually experienced males do not always investigate first, but some instead directly proceed to mounting the estrous female. Additionally, it is possible that anogenital licking could potentially contribute to the stimulation of Fos expression, as this is often observed in males following intromissions and ejaculations.

5.7 Summary and Conclusions

In summary, the morphology of the mitral and granule cell layers, as well as the somata in the mitral layer, is fully masculinized in the TFM affected male rat. However, we detected differential activation of the mitral and granule cell layer between the TFM affected and wild type males, suggesting decreased GABA-ergic tone in the AOB of the TFMs. This decreased output may be used to explain phenomena related to mounting behaviour observed during sexual behaviour tests in the TFMs.

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Figure 29 Schematic diagram displaying the accessory olfactory bulb (AOB) and vomeronasal organ (VNO). Anatomically, the AOB is made up of the glomerular layer, mitral cell layer, and granule cell layer. 'OE': olfactory epithelium.
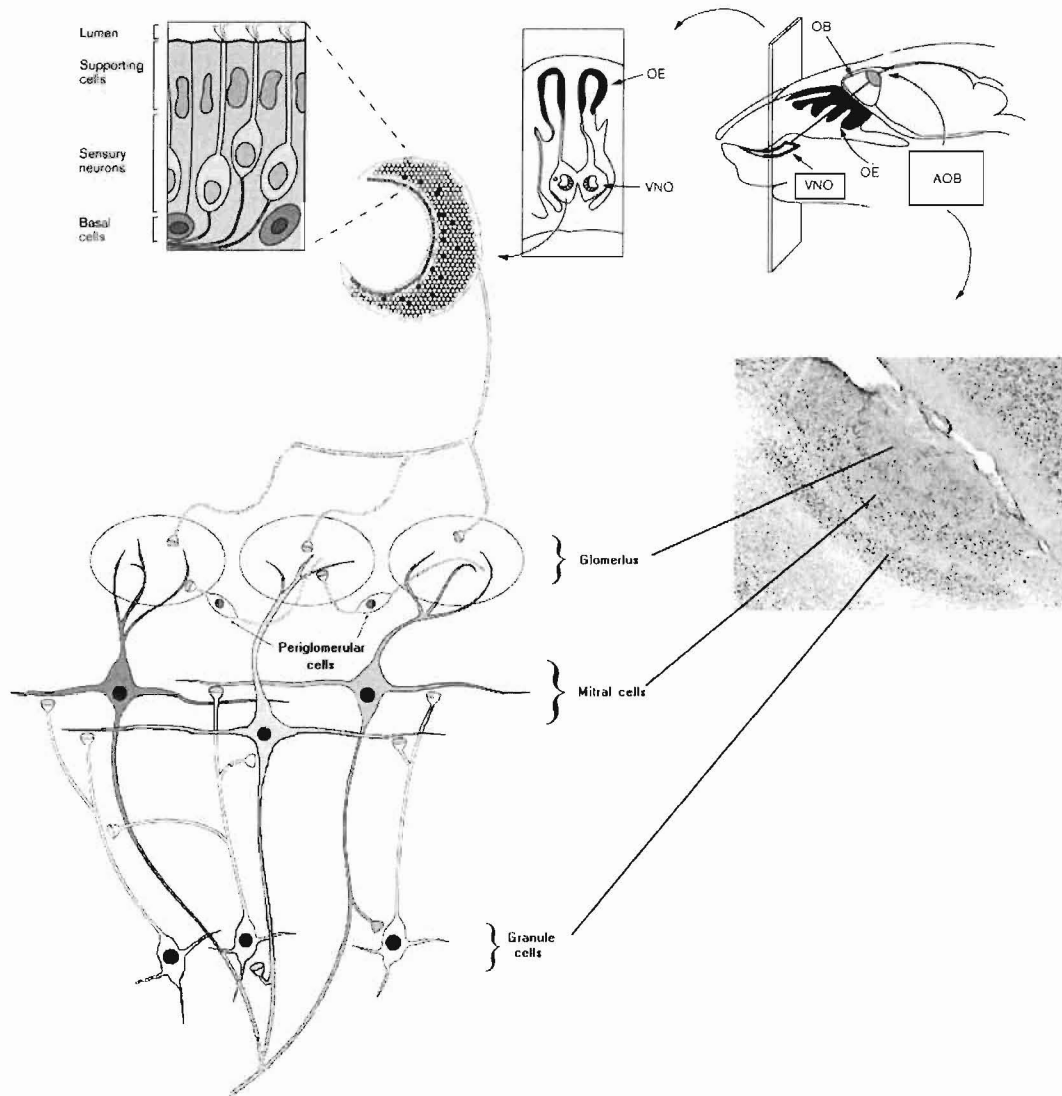
Figure 30 a and b Mean number of Fos immunoreactive cells in the mitral and granule cell layer of WT and TFM affected males. Figure 2B Calculated ratio of activated cells (represented as a percentage) in the mitral cell layer to the granule cell layer. Note: * indicates a significant difference between the groups (see text for details).
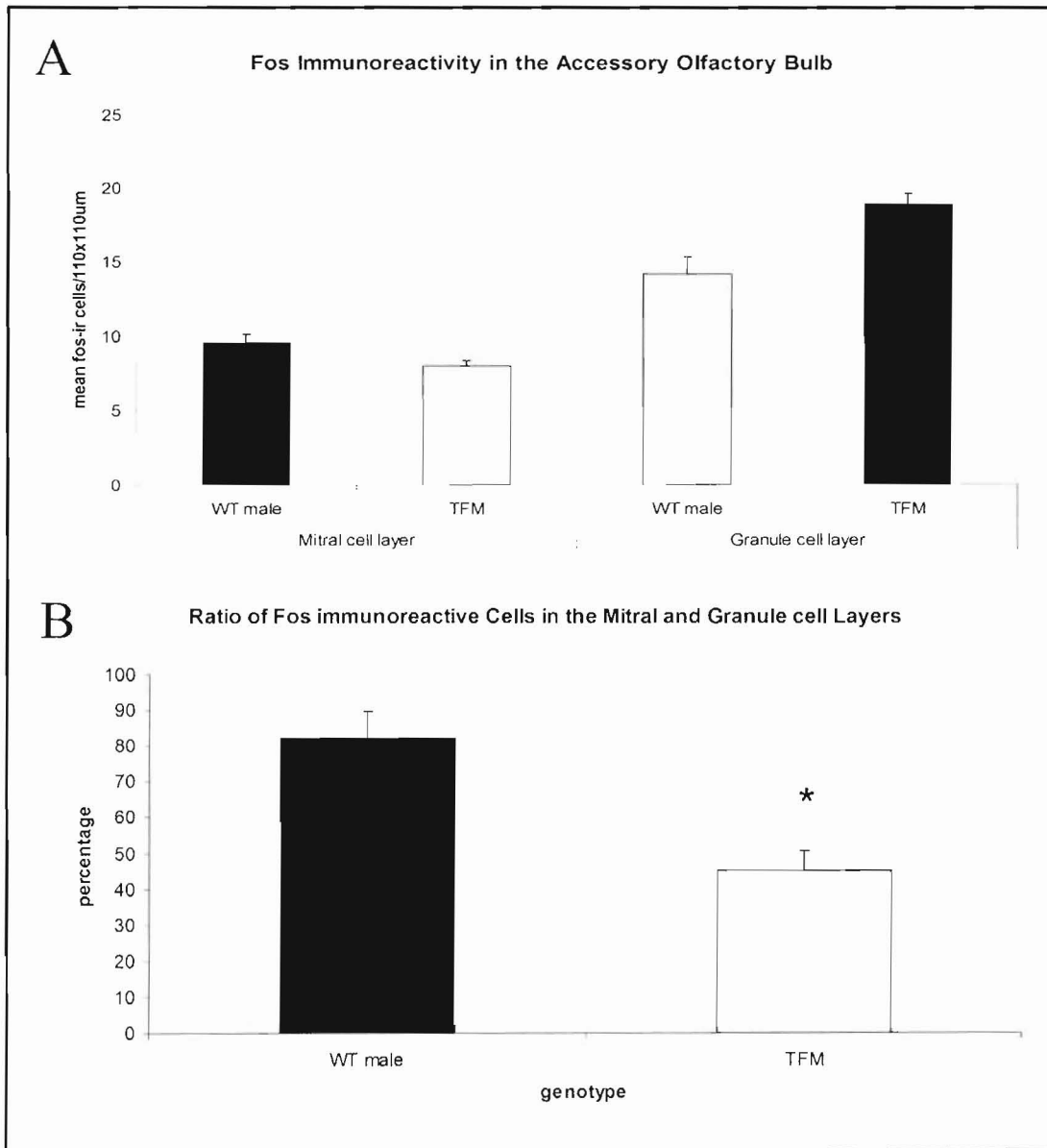
Figure 31 Representative photomicrographs of thionin stained cross sections of the accessory olfactory bulbs in wild type (WT) males and males carrying the testicular feminization mutation (TFM). Note: scale bar= 500μm (with a 2x objective) and 50μm (close up, 20x objective); mcl= mitral cell layer, gcl= granule cell layer.
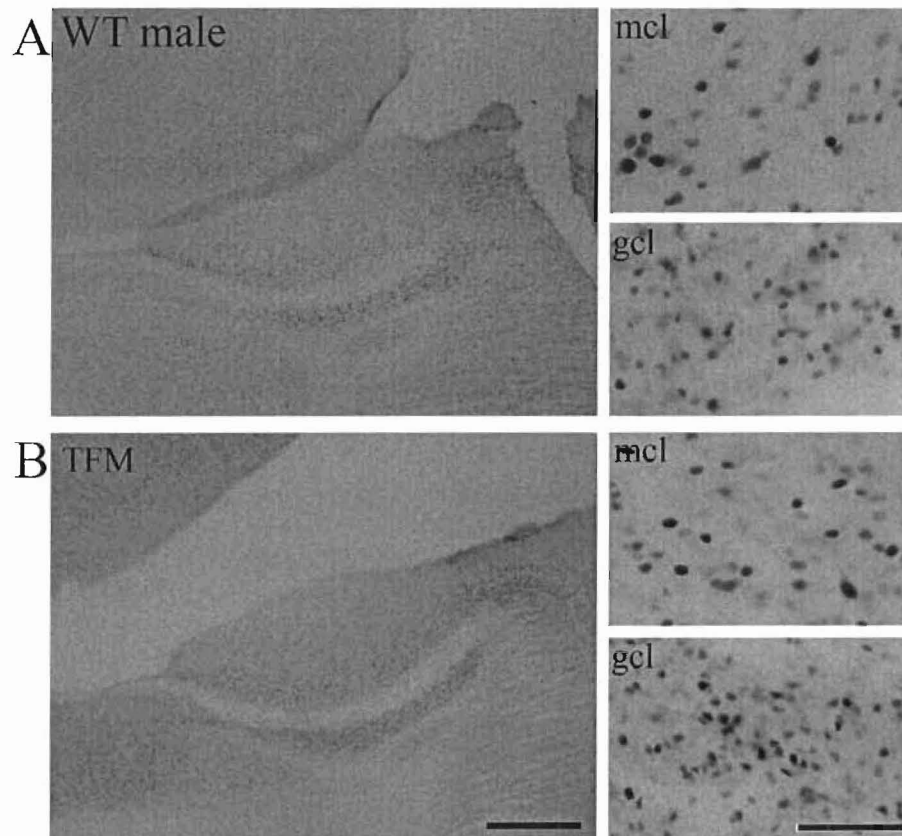
Figure 32 Histograms displaying the average area of the glomerular, mitral, and granule cell layer (top) in WT males, TFMs, and WT females, and the average soma size in the mitral cell layer (bottom). Note: * indicate significant difference at the 0.05 level (s see text for description).
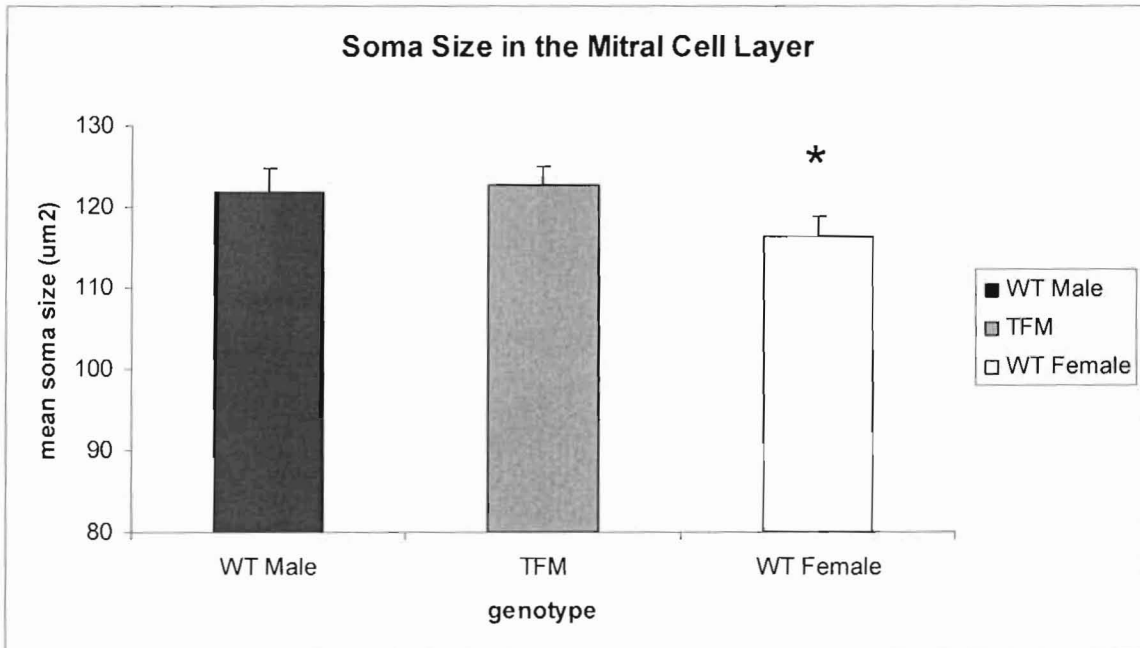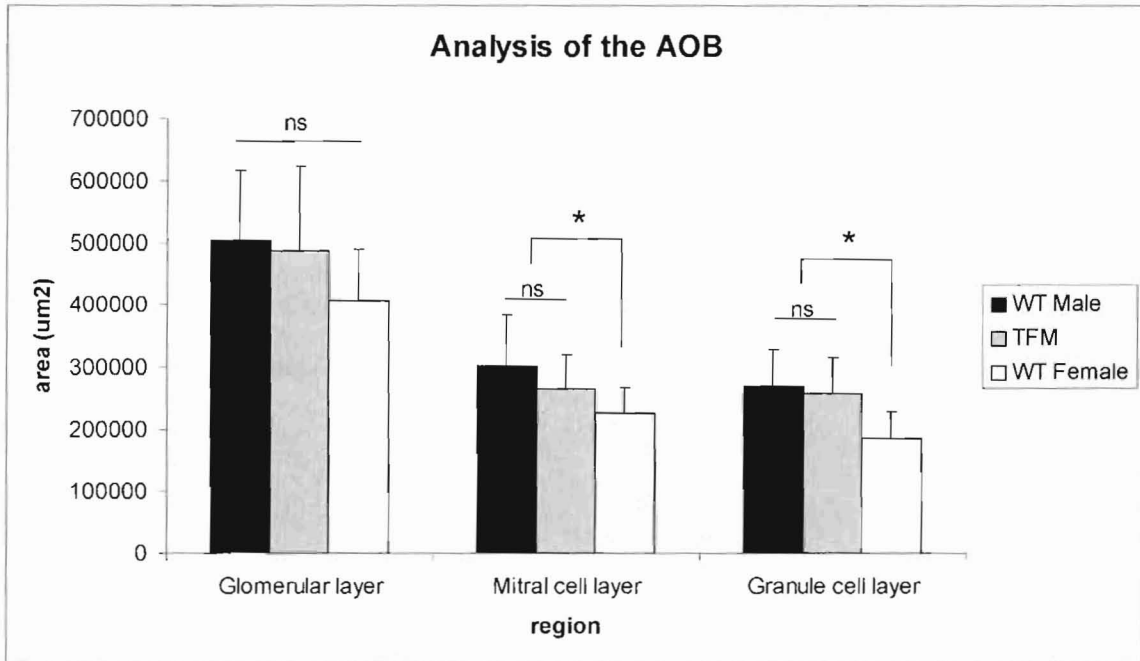
Figure 33 Representative photomicrograms of (A) sagittal sections of the AOB in WT males, TFMs, and WT females., and a (B) close up of the mitral cell layer. Note: scale bars: A=500μm; B=100μm.
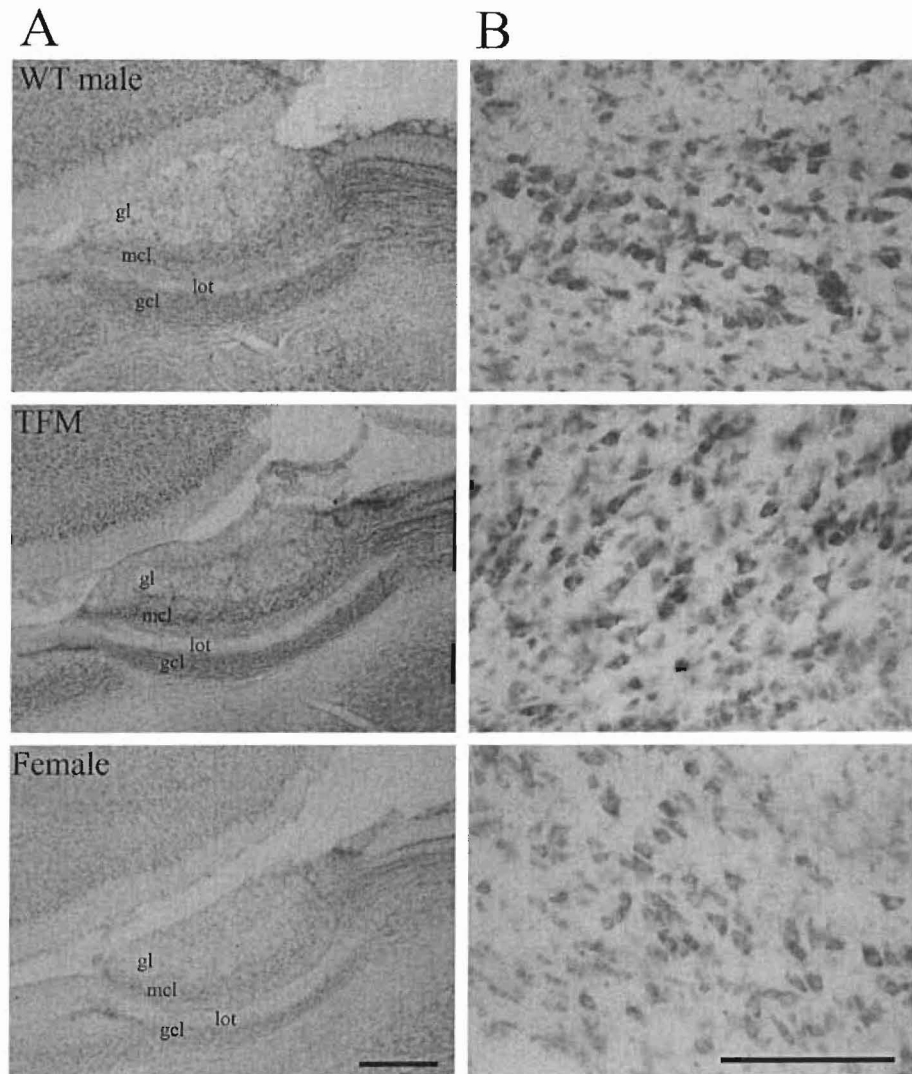
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CHAPTER 6 50 KHZ ULTRASONIC VOCALIZATIONS IN RATS CARRYING THE TESTICULAR FEMINIZATION MUTATION: POSSIBLE IMPLICATIONS FOR MASCULINE SEXUAL BEHAVIOUR

6.1   Abstract:

Fifty kilohertz ultrasonic vocalizations (50 kHz USVs) signal the readiness of the male to mate and increase sexual arousal in females. Testosterone (T) has been linked to USV production as castrated male rats have been reported to produce fewer 50 kHz USVs following exposure to estrous or non estrous female rats. It has been observed that male rats carrying the testicular feminization mutation (TFMs), which are androgen insensitive, display deficits in aspects of sexual performance (i.e., decreased intromissions and ejaculations). Precopulatory behaviour, such as USVs, has not been explored in these mutant male rats. To better understand the role played by androgens in the production of ultrasounds, we first recorded 50kHz USVs from TFM and wild type (WT) males, paired with, and just after exposure to, female rats in estrous or non cycling. We then reversed this and recorded from females paired with, or just after exposure to, TFM affected and WT males. We also examined the effect of potential pheromonal stimulation on USV production in these animals. In the first test, WT males made more USVs compared to TFMs following exposure to both female groups. In the second test, no differences were detected between the WT males and TFMs when they were allowed to explore the region occupied by the females. In the third test, WT males made more 50 kHz USVs than the TFMs, but only following exposure to the estrous females, when the

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observation tank was not cleaned between test sessions. Few 50 kHz USVs were detected from the estrous females, however, the number of USVs emitted by the non estrous female depended upon previous exposure to a WT male or a TFM. The data support the conclusion that the mutation in the androgen receptor affected the ability of the TFMs to emit masculine levels of USVs. We suggest that the decreased USV production by the TFMs may lead to sexual performance deficits through an inability to enhance the receptivity of the estrous female.

6.2   Introduction

Rats emit primarily three kinds of ultrasonic vocalizations (USV) of differing duration and spectrographic structure (as reviewed in Brudzynski, 2007). Pups emit USVs of varying frequency and duration when isolated from their mother, and it is suggested that given the structure of the calls (i.e., frequency modulations), dams have an easier time locating and attending to pups that have gone astray. Alarm calls are emitted by adult rodents in situations that are deemed stressful or dangerous, and thus are thought to communicate a negative emotional state. These USVs are detected within the 18-35 kHz range (often referred to as 22 kHz USVs) and are of long duration (approximately 300-3500 msecs). Rats also emit USVs when they come in to contact with other conspecifics; these calls are detected within the 35-70 kHz frequency range (often referred to as 50 kHz USVs), are of very short duration (30-50 msecs; sometimes referred to as 'chirps'), and are thought to convey a positive internal emotional state. Interestingly, 22 and 50 kHz calls have been detected in mating circumstances; male rats emit 50 kHz calls when exposed to female conspecifics, which may serve to enhance the arousal of the estrous female (McIntosh, Barfield, Geyer, 1978; Floody and Bauer, 1987). Additionally, males also emit 22 kHz calls following ejaculation (i.e., in the post ejaculatory interval), which possibly serves to ward off male competition.

Sex differences in the type and quantity of USVs have been observed in rat pups in the early postnatal period (Naito and Tonoue, 1987), as well as in adult rats (McGinnis and Vakulenko, 2003), hamsters (Floody, Comerci, Lisk, 1987), and mice (Nunez and Tan, 1984) suggesting a hormonal component to this behaviour. Castrated male rats emit very few 50 kHz USVs, whereas castrated male rats maintained on androgen replacement

therapy emit normal amounts of these USVs when presented with an estrous female (McGinnis and Vakulenko, 2003). Additionally, testosterone or estrogen, implanted directly into the hypothalamus of castrated male mice, restored the display of USVs compared to castrates which received a blank implant only (Nyby, Matochik, Barfield, 1992).

Male rats carrying the testicular feminization mutation display a feminine external phenotype (e.g., nipple line, short anogenital distance, and a blind ending vagina) due to insensitivity to the actions of androgens during a developmental critical period (Stanley, Gumbreck, Allison, Easley, 1973). Androgen insensitivity is due to a mutation in the gene encoding the androgen receptor (Yarbrough, Quarmby, Simental, Joseph, Sar, Lubahn, Olsen, French, Wilson, 1990). Behaviorally, both TFM affected rats and mice display deficits in aspects of consumatory sexual behaviour, such as reduced intromissions and ejaculations (Olsen, 1979; Beach and Buehler, 1979; Ohno, Geller, Young Lai, 1974). However, we have previously observed that TFMs prefer to spend more time with estrous females in partner preference tests and have also observed masculine like mount latencies (Hamson and Watson, unpublished observations), suggesting some aspects of sexual behaviour, such as the motivation to engage in copulatory activity, are masculinized in these animals. To date, though, precopulatory behaviors such as ultrasonic vocalizations have not been examined in the TFMs. Given the importance of the androgen receptor in masculine copulatory activity and USV production, as well as the reported function these calls play in the precopulatory phase, part of the deficit in sexual behaviour observed in the TFMs may be due to an inability to properly arouse the estrous female. In order to examine USVs, we have used estrous and

non estrous females as stimulus animals to elicit ultrasounds from gonadally intact TFM and wild type (WT) male rats. In addition, we have also examined the production of USVs in estrous and non estrous females following exposure to either TFMs or WT males.

6.3   Methods

6.3.1   Animals

Sexually naïve Sprague Dawley (SD) WT males (n=7) and TFMs (n=7) were obtained from our breeding colony. Previously identified carrier females were paired with SD stud males obtained from Charles River laboratories (Saint-Constant, Quebec, Canada). Rat pups were weaned at 30 days of age and separated into their four genotypes- WT males, WT females, carrier females, and TFM affected males- on the basis of phenotypic markers and a PCR-based assay (details contained in Fernandez, Collado, Garcia Doval, Garcia-Falgueras, Guillamon, Pasaro, 2003). For this study, only wild type (WT) females were used; the carrier littermates were kept for future breeding and propagation of the androgen receptor mutation in our colony.

6.3.2   Ovariectomies

Twenty nine sexually naïve females in total were ovariectomized under flurothane anesthesia and aseptic conditions. Sixteen females received subcutaneous interscapular implants of 10mm silastic tubing (1.57mm internal diameter, 3.18mm outer diameter, 10mm in length; Dow Corning, Midland, MI) containing crystalline estradiol benzoate (E2; Steraloids, Chicago, Illanois), with the remaining 13 females receiving a blank implant in the back of the neck just under the skin. Females were given post operative

medication for pain as well as an antibiotic and were allowed to recover for a minimum of 10 days. On behavioural test days, the estrogen implanted females received 500μg of progesterone (Steraloids, Chicago, IL) dissolved in corn oil (0.5cc bolus in total), injected subcutaneously 4 hours before testing, whereas the blank females were injected with the vehicle only (corn oil). All animals were maintained on a reversed 12:12 light dark cycle, with food and water available *ad libitum*. Behavioural testing took place in the colony room under red light illumination approximately one hour after lights out.

Surgical procedures conformed to the standards of the Canadian Committee for Animal Care, and were performed under the supervision of an institutional veterinarian; the experimental protocol was subject to prior approval by the Simon Fraser University Animal Care Committee.

6.3.3 General Experimental Protocol for Eliciting 50 kHz Ultrasonic Vocalizations

6.3.3.1 Experiments 1 A, B, and C.

The experimental design was based on the method of Mcginnis and Vakulenko (2003) with some modifications. Briefly, ultrasonic recordings were made in a glass observation enclosure (10 gallon fish tank) that contained an experimental animal and a stimulus animal housed in an isolation cage. Thus, the experimental animal was separated from physical contact with the stimulus animal by use of the isolation cage, but could still hear, see, and smell this animal. In *experiment 1A*, we manipulated the presence of the isolation cage following removal of the stimulus animals; in this experiment, ultrasonic vocalizations were recorded (see below) from WT males and TFMs (the experimental subjects) while paired with either an estrous or a non estrous female (the stimulus animals) for 5 minutes; the stimulus female was chosen at random.

The stimulus animal was then removed (being careful not to disturb the experimental animal); however, the isolation cage was left in place (cage in). This effectively limited the ability of the experimental animal to interact with the physical location occupied previously by the stimulus animal; we then recorded USVs from the experimental animal for another 5 minutes. In *experiment 1B*, USVs were recorded again from WT males and TFMs paired with an estrous or non estrous female housed in the isolation cage for 5 minutes. However, this time, the stimulus females were removed along with the isolation cage (cage out) following this initial 5 minute recording session. We reasoned that perhaps if the experimental animals could directly sense the pheromones left behind by the stimulus animals that this may have an effect on USV production. Thus, the experimental animals were permitted, in this manipulation, to interact with the physical space previously occupied by the stimulus females, which likely contained important pheromonal cues such as urine, feces, and vaginal secretions from these females. It is also important to note that before a new pair of experimental/stimulus animals were tested, the observation enclosure was thoroughly cleaned with quatricide. However, the effect of not cleaning the cage in between each of these experiments was also examined. In this third test *(experiment 1C)*, the experiments were re-run as described above, however, the observation tank was not cleaned in between each test session. Thus, as each TFM or WT male was tested, the tank contained the urine, feces, and preputial gland deposits of either TFMs or WT males from the preceding testing session. However, the testing arena never mixed pheromones of each of the genotypes. That is, the odour of the TFM-affected males was never mixed with the odour of the WT males, and *vice versa.*

We also manipulated the presence of the isolation cage in a counterbalanced order in this experiment to determine what effects it had on the emission of USVs.

6.3.3.2   Experiments 2 A and B.

In another set of experiments, we examined the effect of exposure to either a TFM affected or WT male on the production of USVs in estrous and non estrous females. These experiments were run in exactly the way as that described above, however, the TFMs and WT males became the stimulus animals, and a new set of sexually naïve females (n=10 estrogen + progesterone treated and n=9 gonadectomized, non-cycling females) were used as experimental animals. For this experiment, the isolation cage was either left in (cage in) or removed completely (cage out), and in between each test session, the observation tank was thoroughly cleaned with quatricide. Manipulating the isolation cage was done in a randomized order to minimize order effects, additionally, the stimulus animals were also chosen at random.

6.3.4   Ultrasonic Vocalization Recordings

All of the ultrasonic vocalizations were recorded using a Mini 2 Bat Detector equipped with an SM2 microphone (Ultrasound Advice, London, UK). This is a heterodyne bat detector with frequency division capabilities, which allows the ultrasounds to be transformed into the human auditory range. The bat detector was tuned to a center frequency of 50 kHz (+/-5 kHz) for these experiments; the microphone has a reported sensitivity of 10 dB at this frequency. The Mini 2 is equipped with an audio output and thus we used a standard acoustic cable (with 3.0 mm plugs) to attach it to the 'audio input' of a laptop computer (Asus W5A). The transformed USVs were digitized using

simple audio recording software (GoldWave v.5.13, St. John's, NFLD) that saved the files in mp3 format. The bat detector microphone was placed on the inside of the tank, suspended over the top of the testing arena (approximately 10 inches) and attached to a Plexiglas lid that was fitted over top of the glass aquarium. The microphone was attached to the bat detector, which was located outside of the observation enclosure, via a 10 m cable.

6.3.5 Quantification of Ultrasonic Vocalizations

A researcher blind to experimental conditions counted the number of calls from the recorded audio files using the playback feature of the GoldWave software. Qualitatively, the transformed USVs sounded like bird 'chirps' and were very distinguishable from other environmental sounds such as rats' footfalls and mechanical room noise. Additionally, to aid in the accuracy of counting, the play back rate of the calls was reduced to half speed using the sound software. A second researcher, also blind to experimental conditions, re-counted a sample of USVs (~10%) as a reliability check; the Pearson product moment inter-rater correlation was 0.98 suggesting good inter rater reliability.

6.3.5.1 Note on Paired Animal Conditions

It should be noted that in the paired conditions, there is no way to determine which animals are emitting the ultrasounds. The equipment used in the experiments presented here consist of a microphone that is not directional, and a bat detector that cannot filter out USVs emitted from one animal or another, or allow us to determine which direction the ultrasounds are coming from (i.e., from the WT male or female, or from the TFM or

female). However, we can deduce the amount of USVs each animal contributes when USVs from the paired condition are compared to the USVs recorded in the alone condition.

6.4 Statistical Analysis

All statistical analyses were carried out using SPSS 14.0. Comparisons of the amount of 50 kHz USVs between WT males and TFMs (paired or alone), or estrous and non estrous females (paired or alone), were carried out using T tests with alpha set at 0.05. For *Experiments 1a* and *1c*, we analyzed the number of USVs within WT males or TFMs as a previous experiment observed a greater number of 50 kHz USVs detected was greater when testosterone treated males were previously exposed to estrous versus non estrous females (McGinnis and Vakulenko, 2003). For these comparisons, one way paired samples T tests were used with alpha set at 0.05. Two way independent samples t tests were used to compare the effect of leaving the isolation cage in versus removing the isolation cage in within the estrous and non estrous females; paired samples t tests were not used as the groups were unequal.

6.5 Results

6.5.1 Ultrasonic Vocalizations in TFM affected and WT Males (Experiments 1a, b, and c)

6.5.1.1 Experiment 1a

We recorded the number of USVs from pairs of either TFMs or WT males with either an estrous or non estrous female. As well, we recorded USVs from WT males and TFMs alone for 5 minutes following removal of the stimulus females. In this first

experiment, the isolation cage housing the stimulus females remained inside the observation enclosure (cage in), as well, the observation tank was cleaned in between each trial. The mean (+/- SEM) number of vocalizations per 5 minute test from both the TFMs and WT males is represented in figure 34.

Statistical analysis revealed more USVs were recorded from the cage containing the WT males (paired or alone) than from the cage containing the TFM affected male (paired or alone), except in the condition when a TFM was paired with a non estrous female (With estrous female present: $T_{11}=2.966$, p=0.013; Without estrous female: $T_{11}=3.07$, p=0.011; With non estrous female present: $T_{11}=2.044$, p=0.068; With out non estrous female present: $T_{11}=3.487$, p=0.006). A previous study observed that the number of USVs detected was greater when testosterone treated males had previously been exposed to an estrous versus a non estrous female (McGinnis and Vakulenko, 2003). Thus we examined the number of USVs emitted in all four test conditions using paired samples t tests. Overall, the number of USVs emitted by the cage containing the WT males (following exposure to the estrous or non estrous females) did not differ ($T_6=1.007$, p=0.18). This was also observed when the number of USVs was analyzed in the TFMs following exposure to the females ($T_6=0.11$, p=0.458).

6.5.1.2   Experiment 1b

Because the isolation cage housing the stimulus female was left in the observation enclosure in *Experiment 1a*, we hypothesized that if both WT males and TFMs could detect the non-volatile pheromones (i.e., urine/feces/vaginal secretions) from the estrous female following removal of the cage via direct investigation of where the stimulus animal was located, then the number of calls made may have been different. For the next

experiment, we again recorded USVs from the pairs of experimental and stimulus females for 5 minutes, we then removed the cage (cage out) at the same time the stimulus female was removed, and recorded USVs for another 5 minutes. Again, the cage was cleaned in between each trial (i.e., when a new pair of animals was tested). Figure 35 represents the mean (+/- SEM) number of calls made when the WT males and TFMs were paired with the stimulus females, and the mean number of calls made when these females were removed.

When the stimulus females were present, the pattern of results was similar to that observed in the first experiment in that WT males (paired with an estrous or non estrous female) made more USVs compared to the TFMs (paired with an estrous or non estrous female), however, this was not statistically significant (with the estrous female present: $T_{11}=0.0.873$, p=0.402; with the non estrous female present: $T_{11}=0.846$, p=0.415). Surprisingly, though, the number of 50 kHz calls detected from the WT males or TFMs following removal of the stimulus females was essentially zero. The difference between the WT males and TFMs was not statistically different (with out the estrous female present: $T_{11}=1.191$, p=0.259; with out the non estrous female present: $T_{11}=0.048$, p=0.962).

6.5.1.3   Experiment 1c

We hypothesized that odors from WT males or TFMs could also affect the number of 50 kHz calls made when the experimental animals were tested in the conditions described above. Thus, in this experiment, the observation enclosure was not cleaned from test session to test session, but was cleaned when the experimental group (i.e., WT male or TFM) was changed, or when the stimulus female (i.e., estrous or non

estrous) being used was changed. This testing protocol more closely matched that used in McGinnis and Vakulenko (2003). The isolation cage housing the stimulus female was either left in place or removed entirely in a counterbalanced manner to reduce order effects.

Interestingly, the manipulation of leaving the isolation cage housing the stimulus females in (cage in) or removing it entirely (cage out) did not affect the number of USVs made by the TFM affected or WT males (data not shown). The groups were thus collapsed and the next analysis examined the effect of not cleaning the test arena on USV production.

The mean number (+/- SEM) of vocalizations made by the WT males and TFMs from *Experiment 1c* is shown in Figure 36. The number of USVs detected was only different when WT males were compared to TFMs following exposure to the estrous female (T_{22}=2.95, p=0.007). All other comparisons were not statistically significant.

6.5.2 Ultrasonic Vocalizations from Estrous and Non Estrous Females (Experiment 2)

For this set of experiments, we recorded USVs from either estrous or non estrous females (the experimental animals) paired with either a TFM affected or WT male (the stimulus animals; contained within an isolation cage) for 5 minutes. Following this, we removed the stimulus animals and recorded USVs for another 5 minutes from the experimental females. Once again, we either left the isolation cage in the observation tank following removal of the stimulus animal (cage in) or removed it altogether (cage out); manipulation of the cage was done in a counterbalanced order to minimize order effects.

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Figure 37 displays the mean (+/-SEM) number of USVs made in these tests without taking into consideration the effect of leaving the isolation cage in the testing arena or removing it altogether. The t tests revealed that the number of USVs emitted from the Estrous/WT male pair did not differ from the Non estrous/WT male pair (T_{17}=1.751, p=0.098). However, following removal of the WT male, the number of USVs emitted by the non estrous females was higher compared to the estrous females (T_{17}=3.779, p=0.004). There were no differences detected in terms of the number of USVs emitted from the estrous female/TFM pair versus the non estrous female/TFM pair (T_{17}=1.83, p=0.086), or from either of these experimental groups following removal of the TFM (T_{17}=0.755, p=0.461).

The paired conditions (i.e., estrous female with either a WT male or a TFM) is the same as the paired conditions from the first set of experiments, thus, this data was not analyzed again. What follows are the results from an analysis of USVs from an estrous or non estrous female following removal of the stimulus WT male or TFM.

When the estrous female was exposed to a WT male, but prevented from interacting with the area previously occupied by this animal (i.e., cage in condition), the mean (+/-SEM) number of USVs emitted was 14 (+/-9.99), but when the cage was removed and the estrous female was allowed access to this area the number of calls was 0; the number of USVs was not statistically different (T_8=1.188, p=0.269). When the estrous female was exposed to a TFM but prevented from access to the area previously occupied by this animal, the mean (+/-SEM) number of USVs was 43.667 (+/-14.32), but when allowed access to this area following removal of the TFM, the number of calls

dropped to 0.5+/-0.5; the difference in USVs was significantly different (T_{10}=3.011, p=0.013) (see figure 37).

When the non estrous female was exposed to a WT male but prevented to access the area previously occupied by this animal (i.e., cage in condition), the mean (+/-SEM) number of USVs was 58.6 (+/-18.66); however, when the non estrous female was able to explore this area following removal of the WT male, the mean (+/-SEM) number of USVs emitted rose to 160 (+/-29.6). The difference was statistically different (T_7=3.016, p=0.02). When the non estrous female was exposed to a TFM but prevented from accessing the area previously occupied by this animal, the mean (+/-SEM) number of USVs emitted was 93.25 (+/-27.7); but when given access to this area, the mean (+/-SEM) number of USVs emitted decreased to 4.8 (+/-3.18). This comparison was statistically significant (T_7=3.597, p=0.009) (see figure 38).

6.6   Discussion

In experiment 1A, we observed that the number of USVs made by TFM affected males was significantly fewer than those made by the WT males following exposure to either an estrous or non estrous female. However, the number of USVs was not different between TFMs and WT males when we removed the isolation cage housing the stimulus animals suggesting the scent of the females had an effect on USV production (experiment 1B). This effect appeared to be somewhat attenuated if we did not clean the observation tank in between each of the individual tests (i.e., in experiment 1C), suggesting the scent from other TFMs or WT males can also affect the production of ultrasounds. Interestingly, when we used the TFMs or WT males as stimulus animals and recorded USVs from estrous and non estrous females (Experiment 2), the genotype of the stimulus

animals had an effect on the production of USVs in the non estrous females. That is, previous exposure to a male resulted in an increase in the number of USVs, but only if the female was permitted to physically interact with the space occupied previously by the WT males. Conversely, the number of USVs decreased in the non estrous females if they could detect that a TFM was initially present.

One previous study which examined TFM sexual behaviour suggested abnormal mating of the TFMs was due to a lack of proper estrogen stimulation in the nervous system (Olsen 1979). However, that study did not assess sexual behaviours before gonadectomy, which is necessary to determine if hormone replacement therapy had any effect. What is more, studies have indicated that the TFMs have normal to elevated levels of serum 17-beta estradiol (Roselli, Salisbury, Resko, 1987) as well as normal levels of estrogen binding sites in the hypothalamus (Olsen and Whalen, 1982); thus, other factors may be affecting the TFMs ability to perform sexually. We suggest that the decreased number of vocalizations emitted by the TFMs compared to the WT males may have consequences for the full display of masculine copulatory behaviour, as the completion of a full bout of mating depends upon cooperation of the receptive female. Pairing female rats in estrus with males that have been rendered unable to make ultrasounds reportedly affects the sexual proceptivity and receptivity of the female rats (White and Barfield, 1990; White, Cagiano, Barfield, 1990). In these studies, the females that did not hear USVs from males were more likely to move away when a mount was attempted, and were less likely to remain in the lordosis posture long enough for males to intromit and ejaculate. Additionally, male ultrasonic vocalizations stimulated calls from female rats (White, Gonzales, Barfield, 1993), which in turn served to decrease the

darting behaviour of these estrous females long enough for the males to mount. Thus, the sexual behaviour deficits displayed by the TFMs may be partly a function of the inability to properly arouse the estrous female due to decreased calling behaviour. We have previously observed a minority of TFMs that have displayed the intromission and ejaculatory response (Hamson and Watson, in preparation), and this behaviour as been observed in other labs, as well (Beach and Buehler, 1979; Olsen, 1979). Thus, some TFMs are able to complete a full bout of mating and are capable of an ejaculatory response despite the lack of a penis; however, the majority of these mutants do not display full mating behaviour.

Alternatively, TFMs may produce fewer USVs solely as a result of decreased sexual arousal following pairing with the estrous females. However, as previously mentioned, we have observed mount latencies in the masculine range and mounting activity in TFMs, as well as a clear choice for the estrous female over a non estrous female or a wild type male by these animals (Hamson, Csupity, Ali, Watson, submitted); all of which suggest sexual arousal is intact in the TFMs.

Finally, it may also be the case that the spectral characteristics of the 50 kHz USV may be different in the TFM compared to the WT male, suggesting the TFMs are not able to accurately produce USVs. This may be due to differences in the circuitry between TFMs and WT males that regulates USV production or may also be due to differences in terms of the musculature that is necessary to produce the correct ultrasound. While we cannot rule out the second possibility, we have observed the spectral characteristics of the 50 kHz USVs to be similar between TFM affected and WT males using different equipment (unpublished observations).

As mentioned previously, gonadal steroids appear to play a role in the production of USVs. Castration decreases USV production in rats, hamsters, and mice, whereas steroid hormone replacement restores this behaviour. Interestingly, a combination of estrogen and dihydrotestosterone can stimulate USVs in mice (Pomerantz, Fox, Clemens, 1983), suggesting a synergistic action of both of these androgen metabolites on the expression of ultrasounds. This is also the case when it comes to sexual behaviour; estrogen and DHT stimulate maximal sexual behaviour in castrated rats when the two steroids are given together (McGinnis and Dreifuss, 1989). One area in the brain that seems to be important for the production of USVs is the medial preoptic area. Direct androgenic stimulation of the MPOA can elicit USVs in long term castrates (Holman, Hutchison, Hutchison, 1991; Nyby, Matochik, Barfield, 1992; Matochik, Sypos, Nyby, Barfield, 1994; Sipos and Nyby, 1998). This area of the brain expresses both estrogen and androgen receptors (Simerly, Chang, Muramatsu, Swanson, 1990), and is also important for masculine sexual behaviour (McGinnis and Khan, 1997). Thus, it appears that androgens and estrogens regulate the display of sexual behaviour and the production of ultrasonic vocalizations by acting upon a similar neural substrate. Previous results indicate that aspects of the MPOA are affected by the androgen receptor mutation in that TFMs displayed decreased soma size in this region (Morris, Jordan, Dugger, Breedlove, 2005). Thus, one possibility is that the decreased emission of USVs in the TFMs is the result of a lack of gonadal steroid activation during development or in adulthood in this region.

Important to the contention that part of the mating deficit is due to improper or reduced feminine sexual arousal is the observation of a differential USV response by the

females to the odors of TFMs versus WT males. When the non estrous females were prevented from directly investigating the area occupied by the WT male or TFM (i.e., cage in condition), the number of calls did not differ statistically (58.6 vs. 93.25). However, when the female was permitted direct access to the area occupied previously by a WT male, the number of calls increased (to 160 USVs), whereas the number of calls decreased (to about 5 USVs) when a TFM-affected male was previously in the isolation cage. These data suggest the non estrous females were able to discriminate between the two genotypes via investigation of important pheromones, as was reflected in the number of 50 kHz calls that were emitted. We have casually observed a high degree of aggression displayed by estrous females when paired with TFMs in mating tests. We have also observed in our lab that TFMs do not produce the same number of fecal boli compared to their WT male counterparts (unpublished observations). Further, the production and deposition of precopulatory sebaceous scent gland markings reportedly are androgen dependant (Matochik and Barfield, 1991), suggesting TFMs may also produce less of this substance and make fewer markings. Thus, it is possible that the scent profile of the TFMs is different than that of the WT males and this resulted in the attenuation of 50 kHz from the non estrous females when they had been exposed to a TFM.

We hypothesized that the WT males would make more calls following exposure to the estrous females versus the non-estrous females as was found in McGinnis and Vakulenko (2003). In this previous study, only those males which received testosterone (T) implants vocalized at a high rate and only following exposure to an estrous female. Additionally, because T implanted males vocalized the most following exposure to an

estrous female versus a non estrous female, the authors suggested a role for USVs in recognition of mating status of female conspecifics. Geyer and Barfield (1978) have also observed a differential response to female rats that was dependant upon hormonal status. We were not able to fully replicate these findings, however. For example, WT males displayed virtually the same number of calls following presentation of either the estrous or non-estrous female in both *Experiments 1a* (isolation cage left in) and *1b* (isolation cage removed). Only in *Experiment 1c,* where the scent from other WT males was left in the cage (i.e., no cleaning in between each test), was there a trend for more calls following presentation with the estrous female versus the non estrous female. Incidentally, this final test more closely resembled the experimental conditions of McGinnis and Vakulenko (2003) in which the cage where the observations were made was also not cleaned. This may suggest that the scent from other males played a role in regulating USVs and suggests some alternative interpretations of the purpose of the 50 kHz calls.

6.7   Summary and Conclusions

The data presented here suggest that the mutation in the androgen receptor affects the ability of the TFMs to vocalize at male levels as we observed more USVs in experiment 1a when WT males were paired with an estrous female or were alone when compared to the TFMs. Except for the partner absent trials in test 1b, the number of calls displayed a trend in that WT males made more 50 kHz USVs compared to the TFMs. The decreased amounts of 50 kHz USVs may possibly have consequences for sexual performance in the TFMs.

Figure 34 The average number of 50 kHz ultrasonic vocalizations (USVs) in wild type (WT) males
and TFMs in test one (Experiment 1a). For this test, the WT males and TFMs were
placed with either and estrous ('with Estrous) or non estrous female ('with Blank') and
USVs were recorded for 5 minutes; subsequently, the stimulus females were removed
and USVs were recorded for another 5 minutes from WT males or TFMs alone
('without Estrous' or 'without Blank'). The observation tank was cleaned between
each test session and the isolation cage was left in place following removal of the
stimulus female. The p values for each t test are presented (see text for details).

USVs in WT Males and TFMs (Test One)

mean usv

p=0.013
p=0.011
p=0.068
p=0.006

■ WT Male
□ TFM

with Estrous   without Estrous   with Blank   without Blank
Condition
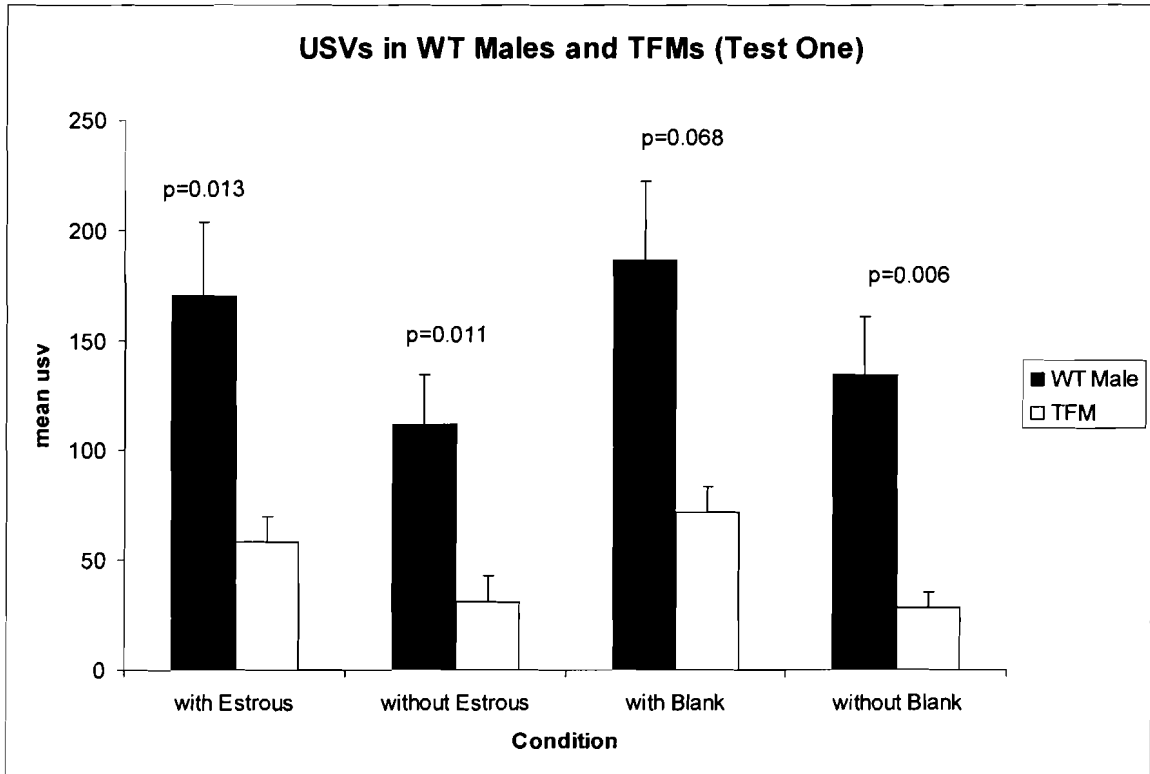
Figure 35 The average number of 50 kHz USVs in WT males and TFMs in test two (*Experiment 1b*).
50 kHz USVs were recorded for 5 minutes from WT males and TFMs paired with
either an estrous female ('with Estrous') or from a non estrous female ('with blank');
subsequently, USVs were recorded for another 5 minutes following removal of the
stimulus females from WT males and TFMs alone ('without Estrous' or 'without
Blank'). The means are higher in the paired conditions for the WT male with the
estrous female and with the blank female, than with the TFM paired with these
females, but this was not significant. In this condition, the cage was cleaned between
each test session, and the isolation cage was removed following removal of the stimulus
female.

USVs in Males and TFMs (Test Two)

193
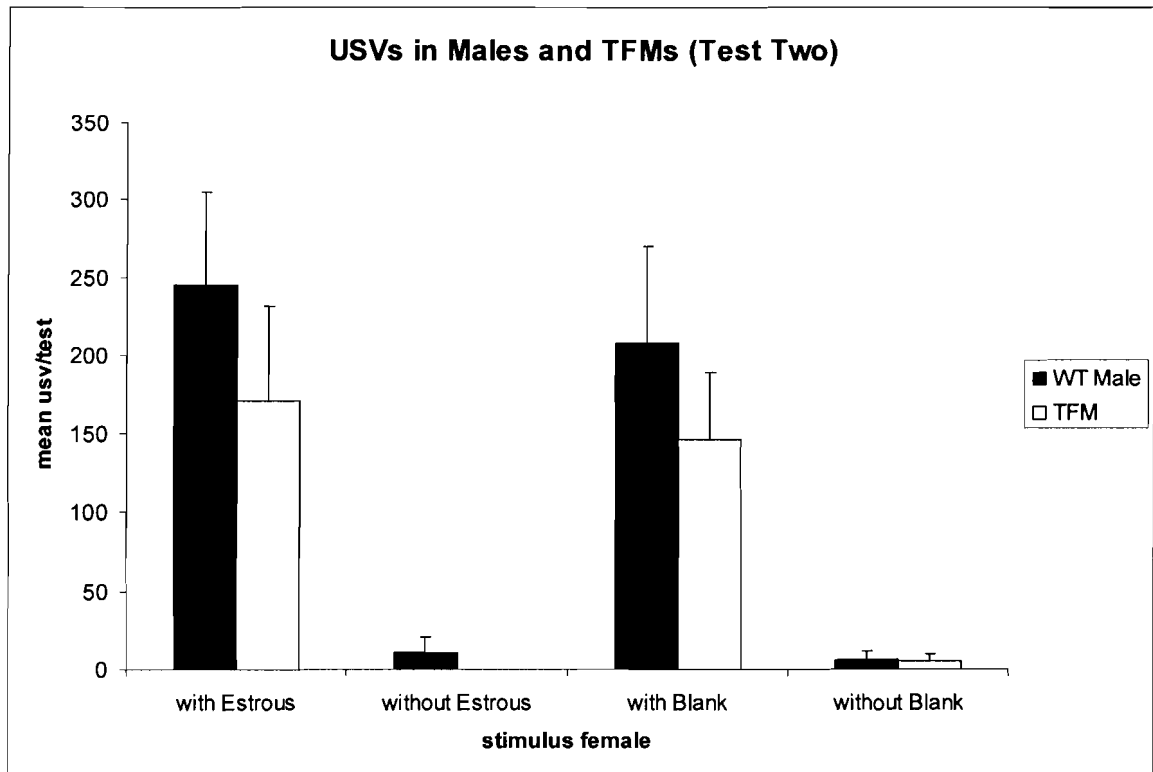
Figure 36 The average number of 50 kHz USVs in WT males and TFMs in test three (*Experiment 1c*).
50 kHz USVs were recorded for 5 minutes from WT males and TFMs paired with
either an estrous female ('with Estrous') or from a non estrous female ('with blank');
subsequently, USVs were recorded for another 5 minutes following removal of the
stimulus females from WT males and TFMs alone ('without Estrous' or 'without
Blank'). For this last test, the observation tank was not cleaned in between each test
session and thus contained the feces/urine/preputial gland discharge from either WT
males or TFMs; manipulation of the isolation cage did not have an effect in this final
test. The number of 50 kHz USVs differed between WT males and TFMs following
removal of the estrous female ('without Estrous; p=0.009) (see text for details).

USVs in WT Males and TFMs (Experiment 3-Groups Combined):
Effect of Not Cleaning the Cage
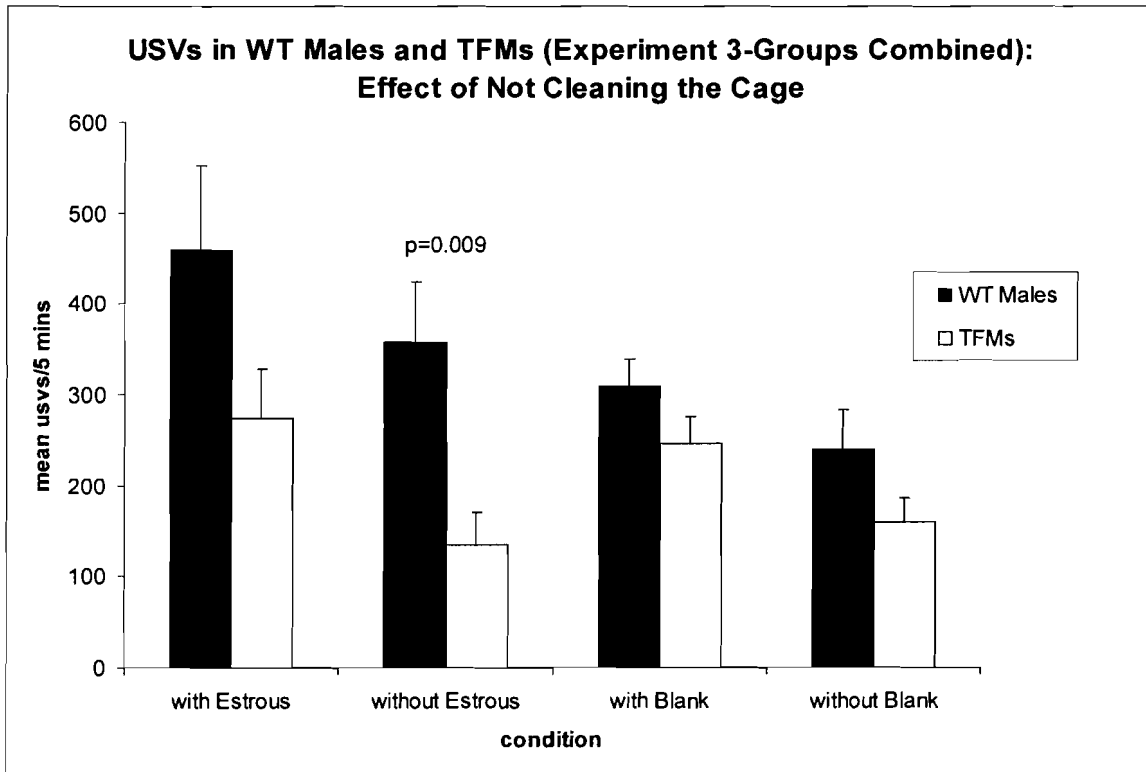
Figure 37 Mean number of USVs from estrous and non estrous females. The data presented here does not take into account leaving the isolation cage in or removing it following presentation with the stimulus animal. Note: *=significant comparison (see text for details).

USVs in Estrous and Non Estrous Females

195
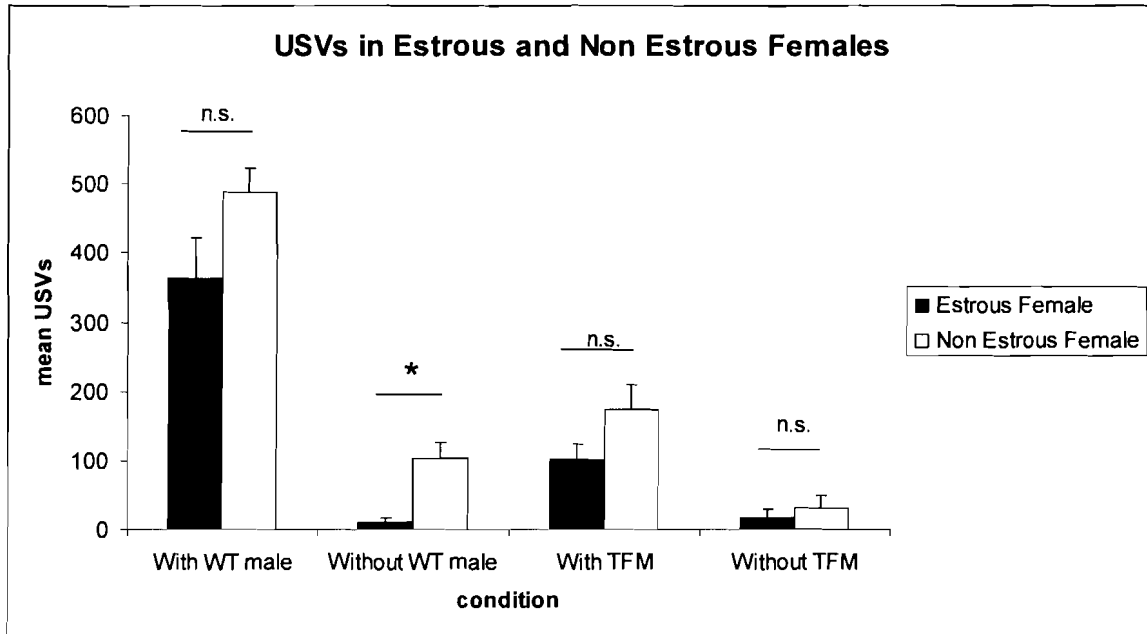
Figure 38 Mean number of USVs from estrous (EOVX) and non estrous (BOVX) females following removal of the WT male or TFM. Removing the isolation cage along with the stimulus animal affected the amount of USVs emitted; the non estrous females increased the number of USVs following presentation of a WT male if allowed access to the area previously occupied by this animal, whereas the number of USVs decreased if previously exposed to a TFM. The estrous females appeared to decrease the number of USVs if allowed to investigate the area occupied by either a WT male or TFM, but the number of USVs was only significantly different following presentation of a TFM. Note: n.s.= non significant comparison.
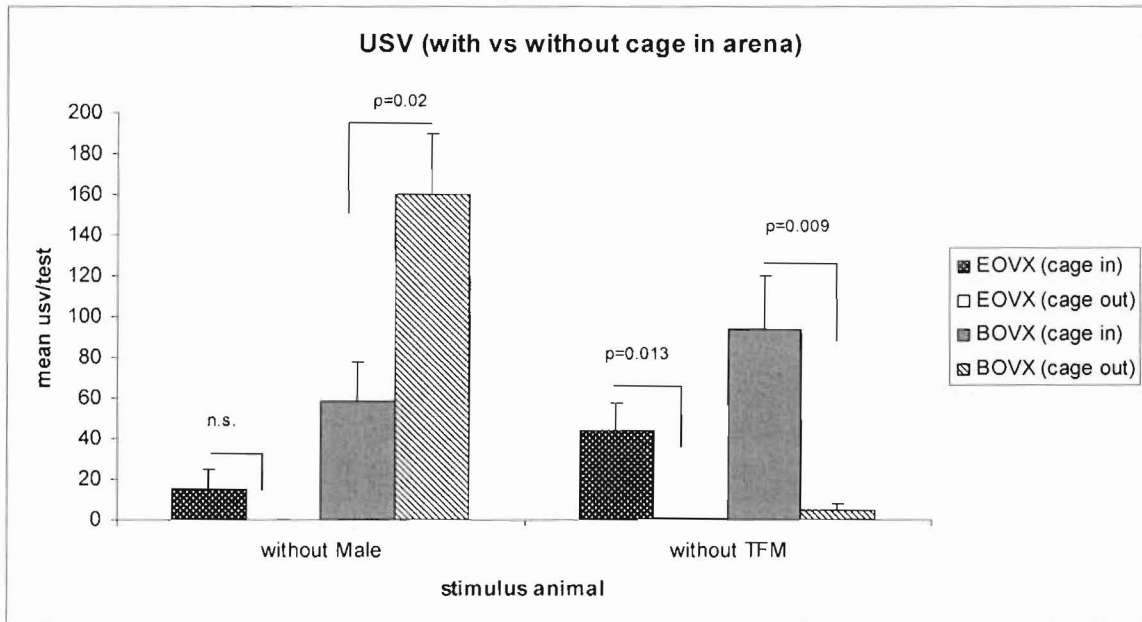
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CHAPTER 7 FOXP2 IMMUNOREACTIVITY IN THE PURKINJE CELL LAYER OF THE CEREBELLUM IS SEXUALLY DIMORPHIC: A RELATIVE OPTICAL DENSITY STUDY

7.1 Abstract:

FOXP2 is a member of the forkhead/winged helix family of transcription factors and has been linked to speech production in humans and ultrasonic vocalizations (USVs) in mice. For example, members of the British KE family who have inherited a mutant FOXP2 allele (point mutation in the DNA binding domain) display verbal and orofacial dyspraxia. In mice, a double knock out of Foxp2 results in the elimination of USVs, and is associated with morphological abnormalities in the Purkinje cell layer of the cerebellum. Previous studies also have implicated endocrine factors in the production of USVs in mammals, but the mechanisms involved in this system are largely unknown. We have recently observed that androgen-insensitive male rats (TFM males) emit fewer 50kHz USVs than wild type (WT) males, although both TFM and WT males emit more USVs than estrous and non-estrous females. Because they have a mutation in the androgen receptor gene, TFM-affected males are an excellent animal model to examine androgenic regulation of gene expression, such as Foxp2 protein expression. In order to establish a possible molecular basis for the hormonal regulation of USV production, we therefore examined Foxp2 immunoreactivity in the caudate/putamen (CPU) and cerebellum of gonadally intact WT males, TFM-affected males, and non-estrous (OVX) vs. estrous (OVX + EB and P) females. Interestingly, analysis of relative optical densities

(RODs; a rough indication of protein expression levels) indicated a sex difference in the cerebellum, in which WT and TFM-affected males displayed, on average, darker and more numerous Foxp2 immunoreactive Purkinje cells than both the estrous and non-estrous female groups. Neither WT versus TFM-affected males, nor estrous versus non-estrous females, were significantly different. Further, the RODs and number of Foxp2 expressing cells in the CPU did not differ between any of the groups, and no differences were detected in the cross sectional area of this nucleus. These differences observed between XY and XX genotypes suggest a possible hormonal component to the expression of Foxp2 in the cerebellum but not the CPU.

7.2   Introduction:

The gene encoding *Foxp2* is highly conserved in structure among different species, with the anatomical distribution of the protein being similar in birds, mice, and humans (Lai et al., 2003). Foxp2 is a member of the Forkhead/Winged helix family of transcription factors (for review see Lehmann, Sowden, Carlsson, Jordon, Bhattacharya, 2003) and has been suggested to be instrumental in the acquisition of language capabilities in hominids. The human version of the FOXP2 protein has undergone selective evolution and differs from our closest relatives, the chimpanzees, in two amino acids, one of which is a serine residue (Enard, Przeworski, Fisher, Lai, Wiebe, Kitano, Monaco, and Paabo, 2002). This change suggests a functional consequence as there is the potential for phosphorylation of this amino acid by protein kinases and may potentially lead to interactions with other proteins or to changes in transcriptional activation (Enard et al., 2002). Interestingly, insertion of the human version of the *FOXP2* into the mouse genome resulted in increased dendritic branching in striatal neurons, changed the structure and emission of ultrasonic vocalizations, and changed exploratory behaviours (Enard, Gehre, Hammerschmidt, Bruckner, Giger,. Holter, Kallnik, Becker, Groszer, Muller, Gailus-Durner, Fuchs, Mouse Clinic Consortium, Klopstock, Wurst, Fisher, Arendt. Hrabe d Aangelis, Fischer, Schwarz, Paabo, 2007). Further supporting a role for this gene in language, mutations in Foxp2 have been observed in men and women of the KE family; affected individuals display deficits in the articulation of the muscles important for speech (orofacial dyspraxia)(Vargha-Khadem, Gadian, Copp, Mishkin, 2005), as well as other linguistic difficulties (White, Fisher, Geschwind, Scharff, Holy, 2006).

The production of ultrasonic vocalizations (USVs) has also been linked to the gene, *Foxp2*, in rodents. A double knock out of *Foxp2* in mice resulted in the elimination of USVs, whereas heterozygotes displayed USVs, but only about half the amount compared to wild type mice (Shu et al, 2005). Morphologically, the mutant mice displayed cerebellar abnormalities, in which the Purkinje cells were irregularly shaped and the molecular layer appeared disorganized (Shu et al, 2005). Morphological abnormalities have also been observed in members of the KE family; reports indicate affected individuals display decreased gray matter thickness in areas of the brain such as the caudate, putamen, and cerebellum (among others; Watkins, Vargha-Khadem, Ashburner, Passingham, Connelly, Friston, Frackowiak, Mishkin, Gadian, 2002; Belton, Salmond, Watkins, Vargha-Khadem, Gadian, 2003), which have been reported to be hypo-activated during word generation tasks (Liegeois, Baldeweg, Connelly, Gadian, Mishkin, Vargha-Khadem, 2003). These areas all express Foxp2 and thus appear to be important in the regulation of speech and ultrasound production.

During the precopulatory phase of sexual behaviour, both male and female rats emit short (20-30 msecs) 50 kHz ultrasonic vocalizations (USVs) which may signal readiness to mate by the male and increase sexual arousal and proceptive displays in the female (McIntosh, Barfield, Geyer, 1978; Floody and Bauer, 1987). Gonadal steroids display both organizational (permanent) and activational (transient) effects upon the production of ultrasonic vocalizations in rodents. Neonatal castration of male gerbils resulted in a feminized pattern of USVs when animals were tested later in adulthood (Holman and Hutchison, 1991a, b), whereas castration of adult male rats resulted in a decrease in the number of calls made when exposed to estrous females or estrous female

odours, and this was reversed with testosterone treatment (McGinnis and Vakulenko, 2002; Matochik and Barfield, 1991). Blockade of androgen receptors with systemic injections of hydroxyflutamide attenuated the ability of androgen replacement therapy to restore 50 kHz calls to precastration levels (McGinnis and Vagell, 1998), suggesting direct involvement of the androgen receptor in the regulation of ultrasonic vocalizations in adult rodents.

One means of studying the effects of androgens on physiology and behaviour has been through the use of animals that are insensitive to the actions of gonadal steroids. Male rats carrying the testicular feminization mutation (TFM) are androgen insensitive due to a spontaneous point mutation in the gene encoding the androgen receptor (Yarbrough, et al., 1990). These TFM-affected male rats exhibit end organ insensitivity to androgens (Naess, Haug, Attramadal, Aakvaag, Hansson, French, 1976), resulting in a feminized external phenotype, internal testis that are androgen secretory (Allison, Chan, Stanley, Gumbreck, 1971), and sexual performance deficits (Shapiro, Goldman, Steinbeck, Neumann, 1976; Beach and Buehler, 1977). In a previous analysis of USV production in the TFMs, we observed a decreased amount of calling behaviour from these mutants following presentation with an estrous female (Hamson, Csupity, Gaspar, Watson, unpublished observations). Additionally, we observed that the number of 50 kHz USVs from both estrous and non estrous females was much lower than that of the WT males when tested under certain conditions. Given that androgens have effects upon USV production and that Foxp2 has been linked with ultrasonic vocalizations in mice (Shu et al., 2005), we sought to determine if a relationship existed between hormonal status and Foxp2 expression in the Purkinje cell layer of the cerebellum. Thus, in the current report,

we examined the intensity of Foxp2 immunoreactivity in TFM affected and WT males, and ovariectomized females given estrogen or blank implants, in the cerebellum, as well as the caudate and putamen. All of these regions express Foxp2, however, only the cerebellum expresses androgen receptor (AR) mRNA, whereas no AR mRNA has been detected in the caudate/putamen. Thus, it was hypothesized that if Foxp2 is associated with USV production, then WT males may display more expression of Foxp2 than the TFMs or either the female group. Furthermore, if sexually dimorphic expression of Foxp2 is mediated by androgen receptor (AR), then dimorphic Foxp2 express should be evident in the cerebellum but not in the caudate/putamen.

7.3   Methods

7.3.1   Animals and housing

Sexually naïve Sprague Dawley (SD) TFMs (n=14), WT males (n=14), estrous females (n=5), and non estrous females (n=5) were used as subjects in the current report. All animals were obtained from our local breeding colony. Briefly, females identified previously as carrying the TFM mutation (carriers) were maited with SD stud males obtained from Charles River laboratories (Saint-Constant, Quebec, Canada). At approximately 30 days of age, the resulting rat pups were weaned and sorted based on external phenotype- short anogenital distances and a lack of a scrotal sac indicated female (carrier and wild type) and TFM genotypes, whereas long anogenital distances and a scrotal sac indicating WT males. The pups with a feminine external phenotype were subsequently ear punched and the tissue was used in a polymerase chain reaction based assay to further distinguish TFMs, carrier females, and wild type (WT) females (details

contained in Fernandez R, Collado P, Garcia Doval S, Garcia-Falgueras A, Guillamon A, Pasaro E., 2003). For this study, only wild type (WT) females were used

7.3.2 Ovariectomies

Females were ovariectomized under fluothane anesthesia and aseptic conditions. Five females received subcutaneous interscapular implants of 10mm silastic tubing (1.57mm internal diameter, 3.18mm outer diameter; Dow Corning, Midland, MI) containing crystalline estradiol benzoate (E2; Steraloids, Chicago, Illanois), with the other 5 females received a blank implant. Females were given post operative medication for pain as well as an antibiotic and were allowed to recover for a minimum of 10 days. On behavioural test days, the estrogen implanted females received 500μg of progesterone (Steraloids, Chicago, IL), subcutaneously in 0.5 ml corn oil 4 hours before testing, whereas the blank females were injected with the vehicle only (corn oil). Animals used in this study were maintained on a reversed 12:12 light dark cycle, with food and water available *ad libitum*. Surgical procedures conformed to the standards of the Canadian Committee for Animal Care, and were performed under the supervision of an institutional veterinarian; the experimental protocol was subject to prior approval by the Simon Fraser University Animal Care Committee.

7.3.3 Foxp2 Immunocytochemistry

Animals were killed by CO2 inhalation and perfused transcardially with 60cc of 0.1M phosphate buffered saline (PBS), followed by 60cc of 4% paraformaldehyde diluted in PBS (4% PFA). Cerebellums were removed and stored in 4% PFA for another 2 hours, and then transferred to 30% sucrose diluted in PBS (w/v) overnight for cryoprotection.

The next day, brains and spinal cords were blocked in the coronal plain, sectioned using a sledge microtome (American Optical) at 50µm into three parallel series, placed in an antigen sparing medium (DeOlmos solution; Watson, Wiegand, Clough, Hoffman., 1986), and stored at -20°C until processed for immunocytochemistry.

One series of the cerebellum sections was processed via the free floating method in multiple custom made tissue wells. Each tissue plate contained a mix of genotypes to ensure differences in staining were not due to processing artifacts. Sections were first blocked for 60 minutes in PBS containing 10% normal goat serum. We used a rabbit polyclonal antibody (Abcam, cat. # ab16046), which is directed against the C-terminus of the human FOXP2 peptide, at a concentration of 1:5000. According to the manufacturer, the sequence recognized by this antibody in humans is predicted to be homologous in the rat, and is specific to Foxp2 (i.e., no homology to other proteins as determined via a search of known or predicted amino acid sequences). Tissue was incubated in the primary antibody (diluted in 0.1 M PBS containing 0.002% v/v Triton X-100) for approximately 18 hours on a shaker tray in the fridge (4°C). The tissue was next incubated in a solution containing a goat anti-rabbit secondary (1:400; Vector Laboratories) diluted in PBS with 1% normal goat serum. Following this, the tissue was incubated in an avidin-biotin complex containing horseradish peroxidase (1:1000; Vectastain Elite Kit, Vector Laboratories) diluted in PBS. Labeled sites were visualized via a diaminobenzidine–hydrogen peroxide reaction; diaminobenzidine was diluted in Tris buffer (pH 7.4; room temperature) with 0.03% w/v NiCl added for intensification. Between all incubations, sections were washed three times for five minutes in 0.1M PBS. Sections were mounted on gelatin coated slides, dehydrated, and cover slipped in

preparation for microscopy. A Sony DXC-950 3ccd camera attached to a Nikon Eclipse E600 (Nikon, Mississauga, Ontario, Canada) microscope was used to collect images of the cerebellar Purkinje cell layer viewed at a magnification of 300x, as well as images of the caudate/putamen (CPU) region in the forebrain viewed at a magnification of 30x for the area analysis and 300x for optical density analysis. Images were loaded into an image analysis program (AIS-MCID, Imaging Research, St. Catherine's, Ontario, Canada) and converted to gray scale (8-bit encoding). Using software define region of interest (ROI) of 13x12 pixels, which roughly corresponded to the diameter of the individual Purkinje cells at this magnification, we measured the optical density of the Purkinje cells relative to the optical density of the tissue exactly adjacent to each cell (i.e., the granular cell layer which contained background staining only). A minimum of 16 cerebellum sections per animal were chosen at random by a researcher blind to the identity of the animals. Every cell expressing Foxp2 immunoreactivity in the outer Purkinje cell layer of the collected images was analyzed. In total, 5662 Purkinje cells from WT males, 6621 cells from TFMs, 5621 cells from ovariectomized females given estrogen and progesterone, and 5827 cells from ovariectomized females given blank implants were analyzed. Optical densities of Foxp2 staining were expressed relative to the optical densities of the adjacent area as per the method of Osborne et al. (2006).

For the caudate and putamen, cross sectional area was calculated on a random subset of slices displaying Foxp2 immunoreactivity by outlining the entire region of the CPU as defined by Oorschot (1996); data were collapsed by group and are represented as mean pixel area. For the optical density analysis, 3 areas were chosen bilaterally at random and 25 randomly selected Foxp2 expressing cells were sampled for optical

density using a probe that was 20x20pixels. Additionally, areas directly adjacent to these cells were also sampled and the relative optical density (ROD) of nuclear Foxp2 immunoreactivity was calculated. We also counted the number of cells per 0.22 mm x 0.22 mm box in order to give an estimate of the approximate number of cells expressing Foxp2 immunoreactivity in this region of the forebrain.

7.4 Statistical Analyses

All statistical analyses were carried out using SPSS 14.0. Possible differences in Foxp2 intensity (i.e., ROD) were analyzed using a one way ANOVA with alpha set at 0.05. When the ANOVA was significant, subsequent planned comparisons were conducted using one way independent T tests.

7.5 Results

7.5.1 Purkinje Cell Layer of the Cerebellum

Figure 39 displays representative Foxp2 immunoreactivity in the Purkinje cells of each group. The average relative optical density (ROD) in the Purkinje cell layer of the cerebellum per group is represented in figure 40. The ANOVA revealed an overall effect of genotype on ROD ($F_{3,34}=6.671$, $p=0.001$). Based on the ultrasonic vocalization data we observed previously, we suspected the WT males would display more Foxp2 immunoreactivity (i.e., higher RODs) than the TFMs and WT females (irrespective of estrous status). As expected, WT males differed from estrous and non estrous females on this measure ($t_{17}=3.848$, $p<0.001$, $t_{15.295}=3.2$, $p<0.001$, respectively). Interestingly, TFM affected males also showed a masculine pattern of expression, differing significantly from either estrous or non estrous females ($t_{16.599}=4.8$, $p<0.001$; $t_{13.857}=4.45$, $p<0.001$,

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respectively). There were no differences detected between the estrous and non estrous females regarding RODs (t_8=1.52, p=0.165), and RODs did not differ between groups ($F_{3, 34}$=0.594, p=0.623) suggesting the results were not artifacts of the staining process.

7.5.2   Caudate and Putamen (CPU)

We analyzed the CPU in a subset of the TFM affected (n=5) and WT males (n=5) and compared the RODs to the female groups (estrogen and non estrous). Figure 41 contains representative photomicrographs of Foxp2 immunoreactivity of the different groups; Figure 42 displays the mean ROD in the CPU of the different groups. The overall ANOVA revealed that there were no significant differences in terms of the ROD between any of the groups ($F_{3, 16}$=1.749, p=0.197) in this area. Additionally, there were no significant differences in the number of cells expressing Foxp2 between the groups ($F_{3, 16}$=0.248, p=0.862), or in the total cross sectional area of the CPU itself ($F_{3, 16}$=1.647, p=0.218) (data not shown).

7.6   Discussion

In the current report, we have analyzed the intensity of Foxp2 immunoreactivity in an area that expresses androgen receptors, the Purkinje cell layer of the cerebellum, as well as in an area that does not express androgen receptors, the caudate/putamen (CPU), in order to investigate the relationship between androgens, ultrasonic vocalizations, and Foxp2. We have previously observed the androgen insensitive TFM affected male rat to display fewer 50 kHz ultrasonic vocalizations compared to wild type (WT) males following exposure to an estrous female. Thus we predicted that if a relationship existed between androgens, USVs, and Foxp2, the WT males should display more intense

immunoreactivity in the Purkinje cell layer compared to the TFM affected males or estrous/non estrous females. We did observe a sex difference in the ROD of Foxp2 expressing cells in the Purkinje cell layer of the cerebellum, with WT males displaying more intense RODs (suggesting higher protein content) compared to both the estrous and non estrous females. However, contrary to our prediction, we did not find a difference in ROD between the WT and TFM affected males. Instead, the data suggest Foxp2 expression is associated with sex chromosome content, as both the TFM affected and WT males displayed increased RODs compared to the female groups in the cerebellum. Additionally, as predicted, we did not find a difference in Foxp2 intensity in the caudate/putamen.

To the best of our knowledge, this is the first paper to report a sex difference in the intensity of the Foxp2 protein in the brain (cerebellum) of rats. In a large analysis of the expression of genes in the mouse, Yang, Schadt, Wang, Wang, Arnold, Ingram-Drake, Drake, Lusis (2006), reported a sexual dimorphism in the expression of Foxp2 transcripts in muscle, liver, and adipose tissue, however, no sex difference was detected in the brain. Possible differences in Foxp2 expression may have been masked by the method used in this previous study as the whole brain homogenates were sampled rather than specific regions. One previous report found a sexual dimorphism in the mRNA levels of another forkhead/ winged helix family member, FoxP1, in zebra finches (Teramitsu, Kudo, London, Geschwind, White, 2004). Foxp1 is known to interact with Foxp2, and thus it is interesting to speculate that perhaps this protein is also sexually dimorphic in the rat. Indeed, a previous study indicated that Foxp1 expression was sexually dimorphic in adipose and liver tissues (Yang et al., 2006); interestingly both

Foxp1 and 2 were found to be expressed in higher amounts in females in adipose tissue, whereas males expressed higher amounts of these in the liver (Yang et al., 2006).

With few exceptions, the development of brain sexual dimorphisms that regulate the display of masculine behaviours depends entirely on androgen released from the gonads. For example, masculinization of certain regions within the hypothalamus is testosterone dependant; these gonadal secretions act upon the nervous system to regulate the fate and outgrowth of cells in males (Arnold and Gorski, 1984). These permanent changes in the organization of the nervous system require androgens to act upon the target tissues within a limited developmental window, spanning just before to just after birth in the rat. However, in adulthood, cells that express androgen receptors may show further plasticity in a transient manner. For example, motoneurons in the spinal nucleus of the bulbocavernosus or cells in the medial amygdala express androgen receptors in adulthood and can enlarge or shrink depending on the presence or absence of androgens, respectively (Watson, Freeman, Breedlove, 2001; Cooke, Tabibnia, Breedlove, 1999). The expression of certain proteins can also depend upon androgen signaling developmentally or in adulthood; for example, the expression of the androgen receptor itself is regulated by androgens just after birth and in adulthood, as gonadectomy reduces AR expression (McAbee and DonCarlos, 1999) whereas androgen replacement therapy up regulates AR production (for example, see McGinnis, Mirth, Zebrowski, Dreifuss, 1989). The current results fit with the idea that Foxp2 expression is not dependant upon androgen receptor activity in the caudate/putamen in development or adulthood as no AR mRNA has been reported to be expressed in this area. It is also well known that androgens such as testosterone can be converted to estrogen via the enzyme aromatase

(Roselli, Salisbury, Resko, 1987) and thus there is the potential for an effect of an androgen metabolite on protein expression. A role for estrogen receptors in Foxp2 expression in this area is unlikely as this region reportedly does not express estrogen receptor mRNA (alpha or beta) (Simerly, Chang, Muramatsu, Swanson, 1990; Li, Schwartz, Rissman, 1997) and does not display aromatase activity (Roselli, Horton, and Resko, 1985). Our data are consistent with this view: Foxp2 intensity in the ovariectomized females with estrogen implants did not differ from ovariectomized females with blank implants. Additionally, previous reports indicate TFM affected males have as much (Hamson and Watson, unpublished observations) or more (Roselli, Salisbury, and Resko, 1987) serum 17 beta estradiol as do WT males.

The data in the current report seem to support the notion that androgen receptor activity is not a necessary for the expression of Foxp2 immumoreactivity; no differences were detected between the female groups despite the differences in serum estrogen levels. Additionally, if estrogen was the major androgenic metabolite regulating Foxp2 expression, the ovariectomized females with the estrogen implants should have displayed more dense labeling than all of the other groups as serum E2 concentrations would have been much higher in these animals.

In terms of a hormonally mediated mechanism, this then leaves the possibility of an organizational effect of gonadal androgens, or its major metabolites, on Foxp2 expression in the cerebellum. Reports indicate a sexual dimorphism in hormone titers developmentally, with males having higher serum androgen and estrogen levels compared to females. Interestingly, there appears to be limited expression of estrogen receptor alpha during the first ten days postnatally in the cerebellum (Ikeda and Nagai,

2006), suggesting a possible mechanism for masculinization of Foxp2 expression in both the TFM affected and WT males. That estrogen receptor activation plays a role developmentally in the masculinization of vocalizations is deduced from studies in which knock out of the alpha and beta isoform of the estrogen receptor eliminates the display of USVs in mice (Ogawa, Chester, Hewitt, Walker, Gustafsson, Smithies, Korach, and Pfaff, 2000). There is also the possibility of a chromosomal effect (i.e., non hormone mediated) upon Foxp2 expression as both TFM affected and WT males did not differ in staining intensity but both differed from the two female groups; the only difference is the sex chromosomal makeup with TFMs and WT males containing a Y chromosome whereas the females do not. To demonstrate, sex difference in vasopressin immunoreactivity has been previously reported in the lateral septum (De Vries, Rissman, Simerly, Yang, Scordalakes, Auger, Swain, Lovell-Badge, Burgoyne, Arnold, 2002), and this sex difference appears to be independent of the gonadal status of the animal supporting a possible genetic cascade theory (Yang et al., 2006) in the development of this pattern of staining.

We cannot rule out the possibility that Foxp2 expression in other areas of the brain could be involved in the production of USVs; reports indicate destruction of the medial preoptic area affects the display of USVs in rats. Intracranial implants of testosterone in the medial preoptic area have been reported to restore ultrasonic vocalizations in castrated male mice (Sipos and Nyby, 1998), suggesting localized androgen receptor stimulation in this area can support USV production. Finally, Foxp2 has been observed in other areas of the hypothalamus (Ferland, Cherry, Preware,

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Morrisey, Walsh, 2003; and unpublished observations), and thus these areas may also be important for Foxp2 mediated USV production.

We have used a measure of optical density as a rough indicator of protein content. The rationale for using this technique is that it gives greater resolution at the cellular levels in terms of measuring directly where the protein is expressed. This is in contrast to similar methods that also using optical density ratings to compare protein expression levels, such as Western blotting, but often use whole tissue homogenates to sample protein levels. Indeed, if we had used this method to compare our groups, we would have been examining the expression of Foxp2 in the Purkinje cell layer, but also the surrounding tissues such as the granule and molecular cell layers, which also contained faint Foxp2 immunoreactivity (see figure 39). The indirect immunocytochemical technique used in the current report also brings up another issue. Namely, what is the ceiling in terms of the optical density obesered in the tissue in the current report? It may be the case that we did not find a different between WT and TFM males because perhaps the optical densities of the Foxp2 expressing cells in WT males were at maximum values; in other words, we may have made a Type I error. However, we feel this is less egregious than a type two error, and thus we accept that perhaps our estimates of Foxp2 expression in the Purkinje cell layer between WT and TFM affected males may be too conservative. Additionally, this does not negate the sex difference observed between XY and XX genotypes; in fact, the sex difference may also be larger than what we are observing given the argument above.

Foxp2 is expressed in several central and peripheral tissues developmentally such as the lungs, liver, muscle, spinal cord and regions of the brain. In the caudate/putamen,

the ontogeny of Foxp2 mRNA expression is embryonic day 13 in the rat (Takahashi, Liu, Hirokawa, Takahashi, 2003). Foxp2 has also been observed in areas that process visual and sensory information, as well as the Purkinje cell layer in the cerebellum. There is a reported 50% reduction in the overall amount of the Foxp2 protein in adults carrying the genetic mutation in the KE family (Scharff and Haesler, 2005). While the specific function of the wild type Foxp2 protein has not been fully elucidated, studies have shown the protein acts embryonically as a repressor of genetic transcription during lung development (Shu, Yang, Zhang, Lu, Morrisey, 2001). Thus, given the role of Foxp2 as a regulator of gene transcription and that it is expressed in areas important for speech production (such as the cerebellum), this protein may have an important function in shaping the vocal production machinery during a critical period of development. Reports indicate that the type of mutation suffered by individuals in the KE family results in aggregation of FoxP2 in the cytoplasmic compartment of isolated Purkinje cells *in situ* (Mizutani A, Matsuzaki A, Momoi MY, Fujita E, Tanabe Y, Momoi T., 2006), as well, it results in a reduced ability to bind DNA (Banerjee-Basu and Baxevanis, 2004). Because of this abnormal cellular localization and loss of transcriptional activity, Foxp2 may not be able to influence the transcription of important proteins developmentally which may be necessary for the proper display of language in adulthood, the result being the morphological and behavioural deficits of the type observed in the KE family (Lai et al., 2001) and in the Foxp2 knockout mice (Shu et al., 2005). However, Foxp2 may also have an important function during song acquisition in juvenile zebra finches as expression is elevated in Area X during this process (Haesler, Wada, Nshdejan, Morrisey, Lints, Jarvis, Scharff, 2004). Interestingly, Area X has been suggested to be homologous

to the human basal ganglia (caudate/putamen) and thus it has been suggested this region may also function in humans in a similar manner as in birds in the acquisition of learned speech (Scharff and Haesler, 2005). However, a specific role for Foxp2 in adult rats seems somewhat elusive as they do not learn how to emit USVs. While species specific information in the song of birds seems to be conveyed in the complex structure of the vocalizations themselves, in rodents, 22 and 50 kHz USVs seem to only differ in the number of calls in a given unit of time (i.e., frequency). Interestingly, Foxp2 regulates the production of proteins that are involved in the structure and function of neurons, and thus this protein may serve to maintain the USV production machinery in adulthood. Specific excision of the Foxp2 mRNA in adult rats or mice may give insights into how this protein interacts with ultrasonic production.

7.7   Summary and Conclusions

In summary, we have observed a sex difference in the intensity of the Foxp2 protein, in which chromosomally male rats displayed higher RODs in the cerebellum compared to females with an XX complement. Deductively, the pattern of results suggest suspect a hormonal component may be important developmentally for establishing masculine levels of Foxp2 expression in adulthood.

Figure 39 Foxp2 immunocytochemistry in the cerebellar Purkinje cell layer (pcl) of wild type (WT) males, TFMs, estrogen treated (OVXeb), and blank treated (OVXbl) females. Note: scale bar is 50µm; ml= molecular layer; gcl=granule cell layer.
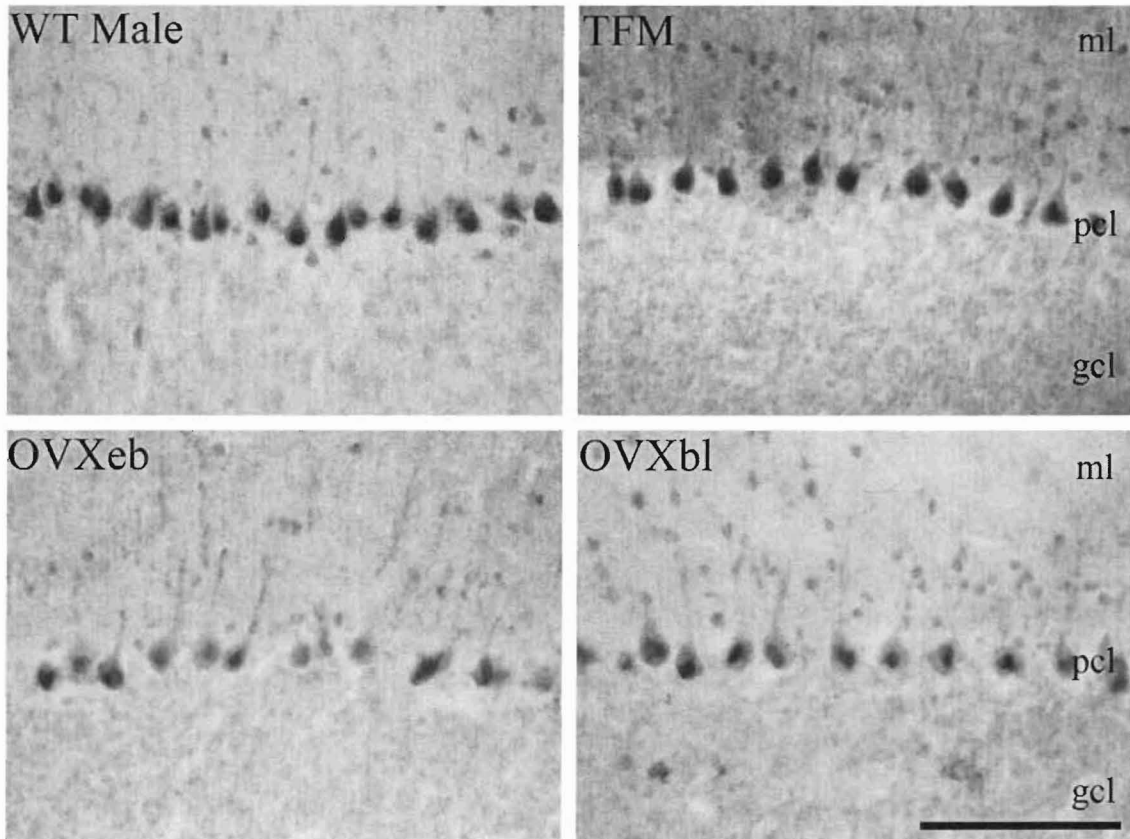
Figure 40 Histograms displaying the Relative Optical Density and number of Foxp2 expressing cells. The p values of the comparisons are reported for the different contrasts made (see text for details). Note: n.s.= non significant.
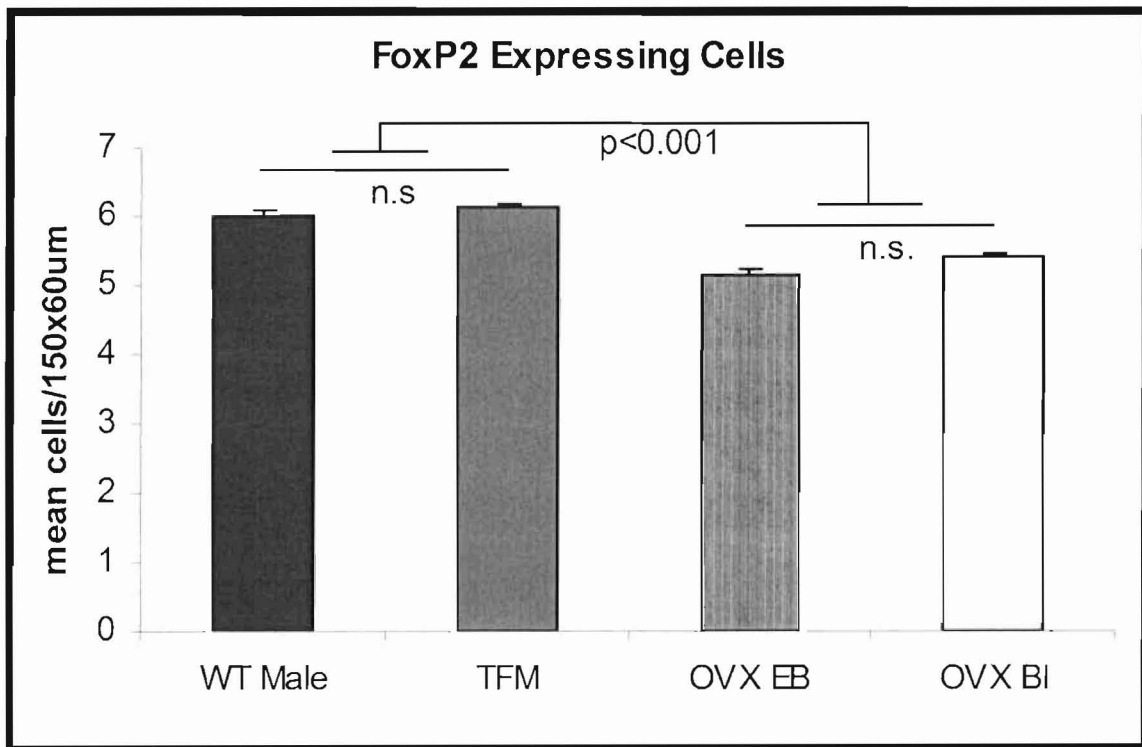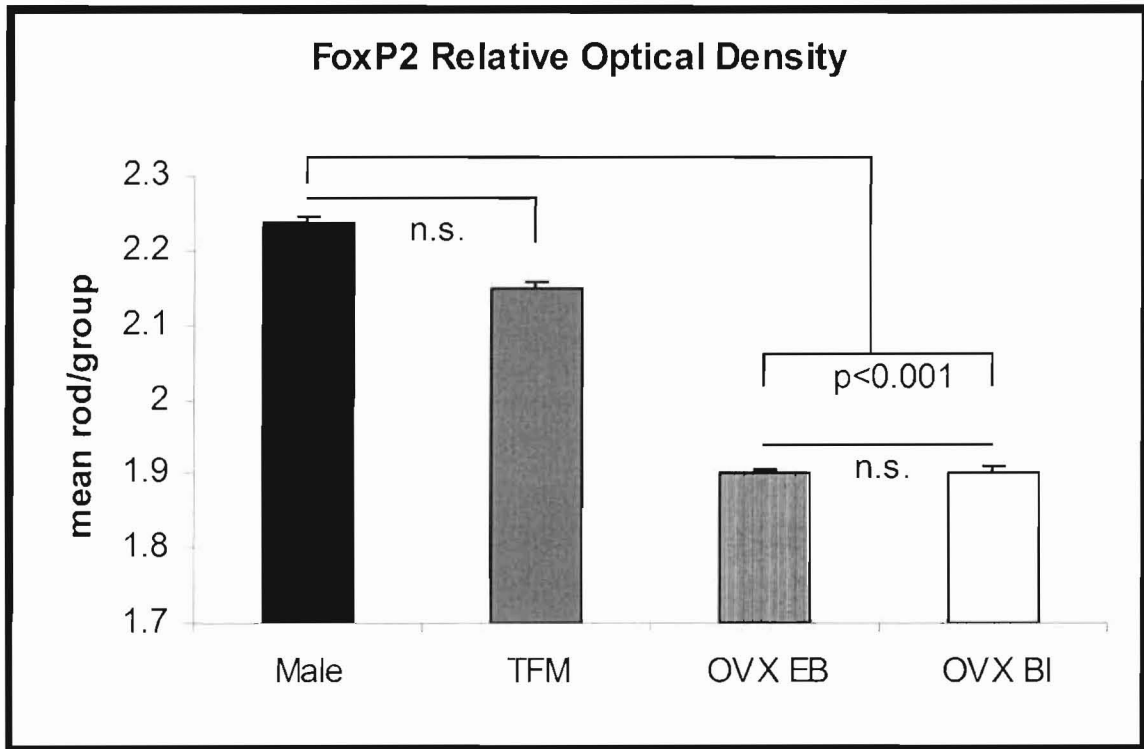
Figure 41 Foxp2 immunoreactivity in the caudate/putamen of WT males, TFMs, estrous, and non estrous females. Note: scale bar= 2mm.
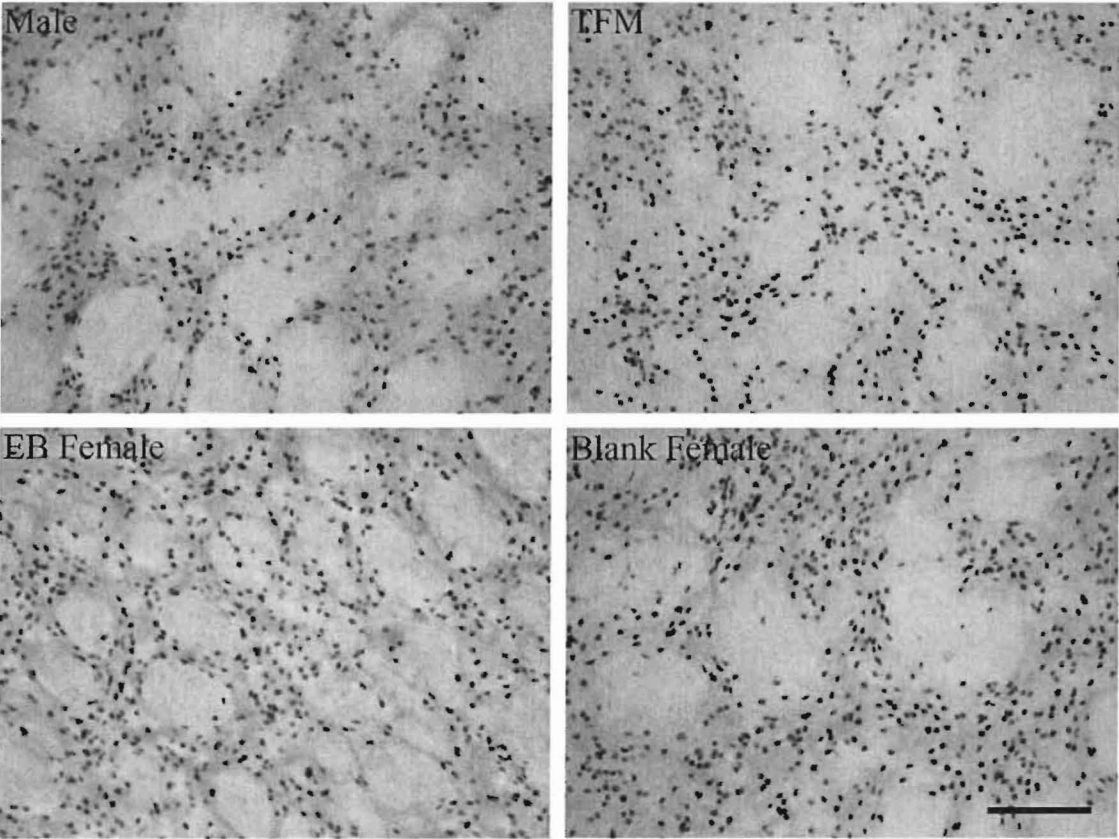
Figure 42 Histograms displaying the ROD of Foxp2 in the caudate/putamen of WT male, TFMs, estrogen treated, and blank treated females. See text for details.
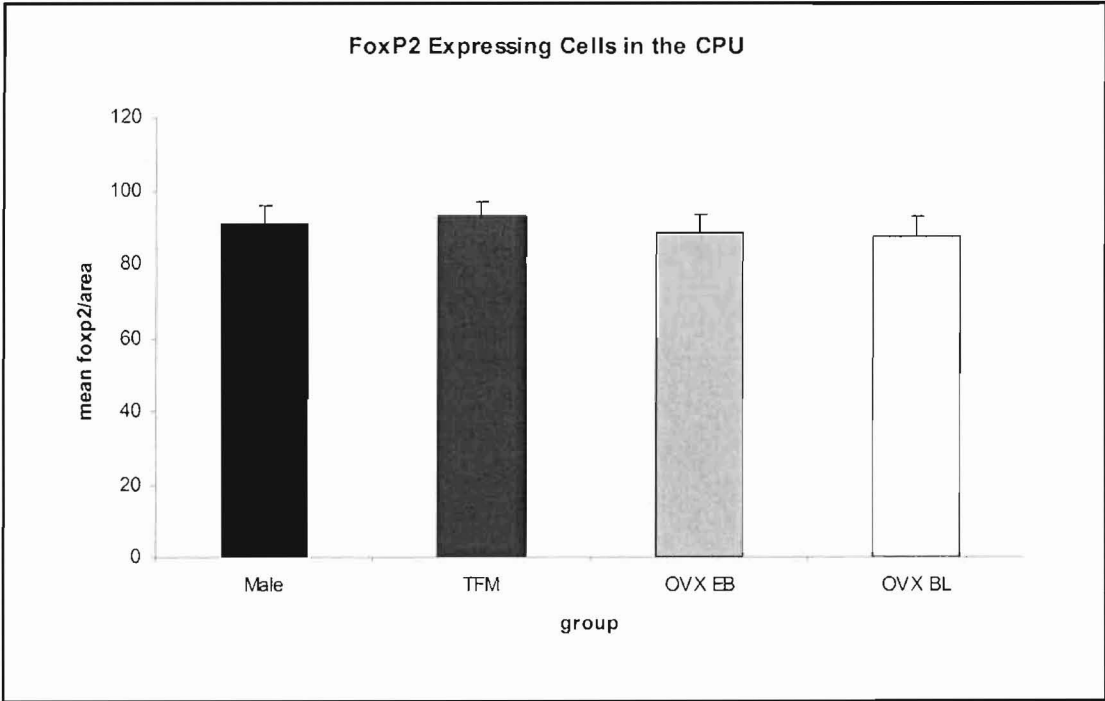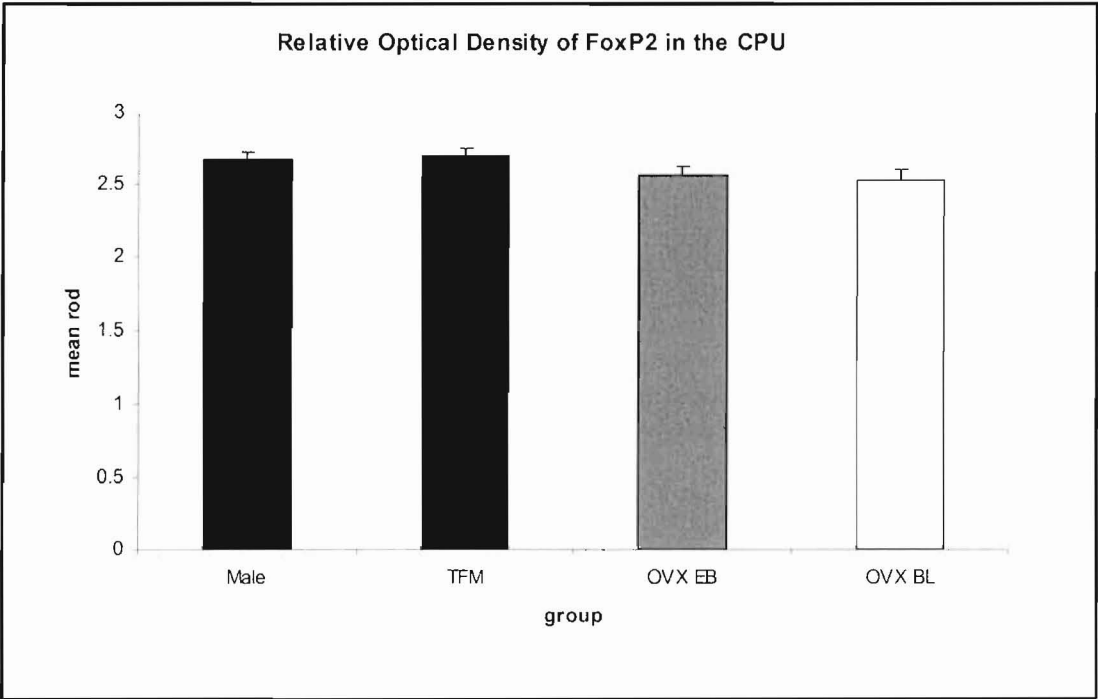
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CHAPTER 8 ESTROGEN RECEPTOR ALPHA IS ELEVATED IN MALE RATS CARRYING THE TESTICULAR FEMINIZATION MUTATION; POTENTIAL CONSEQUENCES FOR MATING BEHAVIOUR.

8.1   Abstract:

Male rats carrying the testicular feminization mutation (TFM) display a decreased

responsiveness to the effects of androgens, such as testosterone. As a result, TFMs

display a feminized external phenotype, such as a nipple line and a blind ending vagina,

and display deficits in copulatory behaviour. Systemic injections of estrogen increased

the display of sexual behaviour in TFMs compared to injections of dihydrotestosterone,

which was ineffective in this regard. Thus, it has been suggested that the ability of

testosterone to be converted into estrogen is compromised in the TFMs, and this is a

possible factor contributing to the lack of sexual performance. However, serum estrogen

levels have been observed to be higher in TFMs compared to WT males, additionally,

radiolabelled estrogen has been reported to be accumulated in the hypothalamus in a

masculine manner. However, these previous studies were conducted before it was

discovered that the nervous system expresses two different estrogen receptors. Thus, in

the current report, we have examined the optical density (a rough indication of protein

levels) of the ER alpha isoform in regions within the brain important for mating in TFMs

and compared them to WT males, and estrogen or blank treated WT females. The

intensity of the ER alpha signal was feminized in the medial preoptic area and

ventrolateral division of the ventromedial division of the hypothalamus, but was

masculine in the medial amygdala. The data suggest that a lack of sexual performance in the TFMs may be due to the abnormal levels of ER alpha in the hypothalamus.

8.2 Introduction

Estrogen receptor α (ER α) belongs to the steroid receptor super-family of ligand activated transcription factors (reviewed in Vasudevan and Pfaff, 2007). Upon binding of 17beta estradiol (E2), ER alpha undergoes a conformational change, and induces transcription of new proteins through interactions with estrogen response elements (EREs) located in the promoter region of estrogen responsive genes. This is the so-called classic genomic response, a characteristic of a number of steroid receptors, which usually takes hours to induce and can last for several days. However, evidence also indicates more rapid effects of estrogen receptor activation; for example, bathing cells in estrogen reportedly results in rapid increases in the activation of adenylate cyclase and cAMP response element binding protein (Aronica, Kraus, Katzenellenbogen, 1994).

The effects of estrogen, however, are not just mediated via the ER alpha, as there is at least one other receptor isoform, ER beta (Mosselman, Polman, Dijkema, 1996; Kuiper, Enmark, Pelto-Huikko, Nilsson, Gustafsson, 1996). ER alpha and ER beta are the products of two separate genes located on different chromosomes (ER alpha is on chromosome six; ER beta is on chromosome 14) (Enmark, Pelto-Huikko, Grandien, Lagercrantz, Lagercrantz, Fried, Nordenskjöld, Gustafsson, 1997), however, the ligand and DNA binding domains are highly conserved between the receptors (Kuiper et al., 1996), and both appear to have an equal affinity for E2. While the expression of these receptors overlaps in certain tissues, binding of E2 and subsequent interaction with

responsive genes containing EREs is not achieved equally by both ER alpha and beta; specificity of action may depend upon the different combinations of repressors and activators each protein recruits (McDonnell and Norris, 2002).

Androgens of gonadal origin play an important role in shaping the nervous system in rodents. Masculinization of the brain requires that androgens such as testosterone, be converted to estrogen via the aromatase enzyme, which in turn activates an estrogen receptor, resulting in an up regulation of protein production; this mechanism of action is encompassed in the aromatization hypothesis (Naftolin, Ryan, Davies, Reddy, Flores, Petro, Kuhn, White, Takaoka, Wolin, 1975; Booth 1977). Evidence supporting the aromatization hypothesis comes from many studies documenting that masculinization of sexually dimorphic cell populations such as the medial preoptic area and parts of the limbic system can be achieved in neonatally castrated male or female rats via estrogen injections given just after birth (Arnold and Gorski, 1984). Exposure to E2 during this critical period masculinizes both males and females for certain aspects of male copulatory activity with a subsequent defeminization of female sexual behaviour such as elimination of the lordosis response (Goy, Phoenix, Gerall, Young, 1959). Additionally, castration of male rats just after birth serves to feminize these animals for copulatory behaviour in adulthood; given the right hormone regime, these males have been reported to display female sexual behaviour (Whalen and Edwards, 1967). Blockade of the aromatase enzyme just after birth blocks the masculinization of male rats for copulatory activity and increases the display of lordosis (Clemens and Gladue, 1978). Importantly, knockout of the gene encoding the ER alpha isoform significantly affects the display of intromissions and ejaculations (Wersinger, Sannen, Villalba, Lubahn, Rissman, De Vries, 1997), as the

proper display of sexual behaviour requires the genomic actions of ER alpha (McDevitt, Glidewell-Kenney, Weiss, Chambon, Jameson, Levine, 2007). While ERαKO male mice display deficits, knock out of the beta isoform of the ER gene does not reportedly affect copulatory activity (Ogawa, Chan, Chester, Gustaffson, Korach, Pfaff, 1999).

Additionally, aromatization of testosterone to estrogen in adulthood is important for the activation of estrogen responsive tissues centrally that regulate mating in males. Blockade of the synthesis of estrogen, via administration of aromatase inhibitors, reportedly affects the display of sexual behaviour (Vagell and McGinnis, 1997). Additionally, direct application of a non steroidal aromatase inhibitor, fadrozole, into the medial preoptic area (Clancy, Zumpe, Michael, 1995), an area critically important for the display of masculine sexual behaviour (Arendash and Gorski, 1983; Liu, Salamone, Sachs, 1997), resulted in an increase in ER alpha immunoreactivity compared to saline treated controls and decreased amounts of mount and ejaculatory behaviour. In gonadally intact male rats given system injections of fadrozole, the number of mounts, intromissions, and ejaculations significantly declined (Huddleston, Michael, Zumpe, Clancy, 2003). However, in similarly treated males given direct injections of E2 into the medial amygdala, the display of mounts and intromissions was not affected, but ejaculations were not different than the injected controls. Together, the data suggest that ER signaling in the medial amygdala is important for regulating the display of mounts and intromissions; however, ER signaling in the medial preoptic area may have a role in the display of the full copulatory response. In female rats, the display or lordosis and sexual receptivity critically depends upon the actions of E2 in the ventrolateral division of the ventromedial hypothalamus (reviewed in Pfaff, Sakuma, Kow, Lee, Easton, 2006).

In male rats, the destruction of the VMH reportedly decreased the latency to mount, and also increased the frequency of certain components of sexual behaviour suggesting the VMH normally inhibits mating in male rats (Christensen, Nance, Gorski, 1977). All of these areas contain androgen and estrogen receptors (Simerly, Chang, Muramatsu, Swanson, 1990), suggesting both hormones can influence their structure and function, as well, they display considerable aromatase activity (Roselli, Horton, Resko, 1985). Finally, these regions have been reported to be activated by sexual behaviour, as these areas display Fos immunoreactivity (a marker of cellular activation) following the display of mounts, intromissions, and ejaculations (Baum and Everitt, 1992; Coolen, Peters, Veening, 1996).

Male rats carrying the testicular feminization mutation (TFM) also exhibit deficits in the performance (i.e., intromissions and ejaculations) aspects of mating (Shapiro, Goldman, Steinbeck, Neumann, 1976; Beach and Buehler, 1977; Olsen, 1979; Shapiro, Levine, Adler, 1980). These animals display a feminine external phenotype (blind ending vagina, nipple line, and short anogenital distance; Bardin, Bullock, Schneider, Allison, Stanley, 1970) that is due to a point mutation in the gene encoding the androgen receptor (Yarbrough, Quarmby, Simental, Joseph, Sar, Lubahn, Olsen, French, Wilson, 1990). This causes an insensitivity to the effects of testosterone and other androgens, which is necessary for the masculinization of the external genitalia during a developmental critical period. While there is residual activity of the mutant androgen receptor (in rats, mice, and humans that carry the mutation; Fox, Blank, Politch, 1983), it appears the TFM rat is non responsive to physiological doses of androgens (reviewed in Bardin and Bullock, 1974). It has been observed that the TFMs contain estrogen binding

sites in the hypothalamus (Olsen and Whalen, 1982), an indirect indication that estrogen receptors are present in these mutants. However, these studies pre-date the discovery of the different estrogen receptor isoform (Mosselman, Polman, Dijkema, 1996) and given the importance for the ER alpha for sexual activity, we decided to specifically examine the ER alpha isoform in areas of the brain important for mating in the TFM rat (i.e., medial preoptic area, medial amygdala, and ventromedial hypothalamus). Thus, in the current report, we have investigated the optical density (a rough indication of protein levels) of the ER alpha isoform in gonadally intact TFM affected males and compared them to both WT male and female rats with differing levels of circulating estrogens. An examination of the ER alpha isoform in the TFM affected male rat may give some insight into the deficit in mating performance these animals display as a lack of the full copulatory response may be due to an abnormality of ER alpha expression and/or distribution in areas critical for mating.

8.3   Methods

8.3.1   Animals and housing

TFM affected males (n=9), WT males (n=9), gonadectomized females artificially brought into estrous (n=5), and gonad intact females in diestrous (n=10) were obtained from our colony of breeding stock females which carry the testicular feminization mutation (known as 'carriers'). Known carrier females were paired with Sprague Dawley stud males obtained from Charles River laboratories (Saint-Constant, Quebec, Canada) in breeding cages to generate the animals. Pups were weaned and separated at approximately 30 days of age based on external phenotype; short anogenital distances and a lack of a scrotal sac suggested female (carrier and wild type) and TFM genotypes,

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whereas long anogenital distances and a scrotal sac suggested WT males. At this time, the pups with feminine phenotypes were also ear punched and the tissue was used in a polymerase chain reaction based assay to distinguish TFMs from wild type and carrier females (details contained in Fernandez, Collado, Garcia Doval, Garcia-Falgueras, Guillamon, Pasaro, 2003). For this study, only wild type (WT) females were used; the carrier female littermates were kept for future breeding and propagation of the androgen receptor mutation in our colony.

8.3.2 Surgery

Five females were ovariectomized under fluothane anesthesia. These females also received subcutaneous interscapular implants of 10mm silastic tubing (1.57mm internal diameter, 3.18mm outer diameter, 10mm in length; Dow Corning, Midland, MI) containing crystalline estradiol benzoate (E2; Steraloids, Chicago, Illanois), at the time of surgery. Animals were given post operative medication for pain as well as an antibiotic and were allowed to recover for a minimum of 10 days. On the day of tissue harvesting, females received 500μg of progesterone (Steraloids, Chicago, IL), subcutaneously in 0.5 ml corn oil 4 hours before they were sacrificed. This hormone regime effectively mimics the endogenous estrogen/progesterone serum levels observed in female rats in behavioural estrous. Surgical procedures conformed to the standards of the Canadian Committee for Animal Care, and were performed under the supervision of an institutional veterinarian; the experimental protocol was subject to prior approval by the Simon Fraser University Animal Care Committee.

8.3.3 Diestrous Determination

To minimize the number of ovariectomies, diestrous was determined in 10 gonad intact females. For this procedure, stud males were briefly placed in the home cage of the females and at the first sign of receptivity (ear wiggling and hop darting), which indicated these animals were in behavioural estrous, the males were immediately removed to prevent pseudo pregnancy. Females' estrous cycles were then tracked using this method for a minimum of 8 days (2 cycles). Only females displaying 4 day cycles were used and were sacrificed 2 days following the display of sexual receptivity (i.e., when serum estrogen was low).

All animals used in this study were maintained on a reversed 12:12 light dark cycle, with food and water available *ad libitum*.

8.3.4 Estrogen Receptor alpha Immunocytochemistry

Animals were killed by CO2 inhalation and perfused transcardially with 60cc of 0.1M phosphate buffered saline (PBS), followed by 60cc of 4% paraformaldehyde dissolved in PBS (PFA). Brains were removed, post fixed in 4% PFA for 2 hours, and then transferred to a 30% sucrose/PBS (w/v) solution over night for cryoprotection. The following day, tissue was blocked in the coronal plane and sliced into 50μm sections using a sledge microtome (American Optical). Every third section was collected, placed in an antigen sparing solution, DeOlmos (Watson, Wiegand, Clough, Hoffman, 1986), and stored in the freezer until processed immunocytochemically (ICC).

ICC was carried out using the free floating method in custom made tissue wells and trays. Each tray contained two genotypes to ensure differences in staining were not due to processing artifacts. All incubations were carried out at room temperature on a shaker

tray unless otherwise stated. Washing steps (three x five minutes) between the incubations was carried out with PBS containing 0.1% triton X100 (PBSx). Tissue was first blocked with PBSx containing 10% normal goat serum (NGS) for one hour followed by a one hour incubation at 4°C of a rabbit anti-ER alpha IgG (C1355 diluted at 1:5000; Upstate Biotech) diluted with PBSx and containing 1% NGS. According to the manufacturer, the ER alpha primary antibody binds specifically with the ER alpha protein and does not cross react with the highly related ER beta isoform. The antibody recognizes the last 15 amino acids of the protein (TYYIPPEAEGFPNTI) and a search of related proteins using a basic local alignment search tool (BLASTp) revealed that this sequence does not share significant homology with other amino acid sequences. This suggests that the antibody is specific for the rat ER alpha protein. Additionally, this antibody will recognize bound and unbound ER alpha. Next, the tissue was washed and incubated with biotinylated goat anti-rabbit IgG (B-100; Vector) was used at a concentration of 1:400 and diluted in PBSx containing 1% NGS. An avidin biotin peroxidase complex was then used at a concentration of 1:200 and diluted in PBSx to bind the secondary antibody. Labeled sites were visualized via a diaminobenzidine–hydrogen peroxide reaction; diaminobenzidine was diluted in Tris buffer (pH 7.4; room temperature) with 0.03% v/v NiCl added for intensification. Sections were mounted on gelatin coated slides, dehydrated in a series of graded ethanol solutions, and covered slipped in preparation for microscopy. A Sony DXC-950 3ccd camera attached to a Nikon Eclipse E600 (Nikon, Mississauga, Ontario, Canada) microscope was used to collect images of the medial preoptic area (MPOA), posterodorsal medial amygdala (MeApd), medial dorsal medial amygdala (MeAd), and the ventrolateral division of the

ventromedial hypothalamus (VMHvl) viewed at a magnification of 300x. The images collected were compared to the atlas of Swanson (2004); panels 19 and 20 represented the approximate locations that the ER alpha was assayed; panels 27-30 were used to determine the locations of the VMHvl, the MeAd, and the MeApd. Images were loaded into an image analysis program (AIS, Imaging Research, St. Catherine's, Ontario, Canada) and converted to gray scale (8-bit encoding). The image analysis program allows for the use of 'user defined probes' to measure the optical density of ER alpha expression cells; the probe used was set at 20 pixels in diameter which roughly corresponded to the diameter of the nuclear envelope of ER alpha expressing cells. For this study, the collected images were also magnified 300% using the AIS program to aid in the analysis of the tissue. The optical density of 25 cell nuclei per section was collected by a researcher blind to experimental conditions, as was the optical density of the area directly adjacent to the cell (i.e., background). The absolute optical density of the 'background' was subtracted from the absolute density of the 'nuclear' ER alpha immunoreactivity as per the method of Monks et al. (2001) and Osborne et al. (2006); this procedure 'corrected' for background staining and the final measurement of density is represented in optical density units (ODU).

8.3.5   Measurement of Serum 17 beta Estradiol

For measurement of serum E2, trunk blood was collected from TFM (n=25), WT male (n=15), and ovariectomized females (n=5; implanted with 10mm silastic capsules containing crystalline estrogen) into 1.5 ml aliquots; the WT males, TFMs, and estrous females form this study were combined with blood collected from additional animals from our colony. The blood was left in the refrigerator (4° C) for 72 hours, and then the

serum was extracted by centrifuging the blood at 4000rpm's for 4 minutes and then at 6000rpm's for an additional 3 minutes. Serum was removed, placed in a new aliquot and then stored in the freezer (-20° C). Serum was shipped on dry ice to the Biomarkers Core Laboratory (Yerkes National Primate Research Center; Emory University, Atlanta, GA) where E2 was assayed using a commercially prepared radio immunoassay kit (Diagnostic Products Corp.; Los Angeles, CA). The assay range of this kit is 5.97-1194.74 pg/mL; E2 was assayed from 200ul of serum in the current report. The intra assay coefficient of variance was 7.95% at n=7 replications, whereas the inter assay coefficient of variance was 6.62% when extracted at 70 pg/ml (n=8 replications), 4.64% at 159.63 pg/ml (n=8 replications), and 9.56% at 977.43 pg/ml (n=7 replications).

8.4 Statistical Analyses

All statistical analyses were carried out using SPSS 15.0 for the PC. Mean optical density units were analyzed using an analysis of variance (ANOVA); for ANOVAs significant at the 0.05 level, *post hoc* comparisons were made using a family wise error rate correction, Bonferroni, for multiple comparisons. Serum E2 levels were analyzed using a one way ANOVA with alpha set at 0.05; where appropriate, *post hoc* analysis was made using Tukey's least significant difference statistic.

8.5 Results

8.5.1 Optical Density of ER alpha Immunoreactivity:

Figure 43 (A, B, C, and D) displays the mean (+/-SEM) intensity (represented in optical density units or ODU) of ER alpha cells in the medial preoptic area (MPOA; 43A), the ventrolateral division of the ventromedial hypothalamus (VMHvl; 43B), the anterodorsal

(MeAd; 43C) and posterodorsal (MeApd; 43D) divisions of the medial amygdala. Figures 44, 45, 46, and 47 contain photomicrographs of ER alpha immunoreactivity in the MPOA, VMHvl, MeAD, and MePD, respectively. For each of the areas analyzed, we quantified the absolute optical density of the nucleus of cells expressing ER alpha and the region directly adjacent to the cell (i.e., background). To ensure results were not due to differences in background staining, we also compared the absolute density of the background between the different groups. The ANOVA revealed no significant differences in background staining for each of the areas analyzed (data not shown), suggesting possible differences in intensity were likely due to differences in the optical density of the ER alpha expressing cells themselves and not due to staining artifacts.

In the MPOA, we observed the WT males to display the lowest optical density compared to the other three groups; TFMs, however, displayed feminine values. The ANOVA revealed a significant effect of genotype (F3, 29=12.787; p<0.001), with the *post hoc* tests revealing that the WT males displayed, on average, lower ODUs than the estrous (p=0.008) and non estrous (p<0.001) females, as well as the TFMs (p=0.009) (see figure 44). The TFM affected males displayed similar ODUs as the estrous and non estrous females.

In the VMHvl, WT males and estrous females displayed equivalently low levels of staining intensity compared to the TFM and non estrous females (which also displayed similar values). The ANOVA revealed an overall significant effect of genotype on the ODUs of ER alpha expressing cells in the VMHvl (F3,24=7.844, p=0.001). The *post hoc* tests revealed that WT males displayed lower ODUs than the TFM affected males (p=0.02) and the non estrous females (p=0.001), however, WT males and estrous females

displayed similar ODUs (p=1.00) (see figure 45). The estrous females displayed lower ODUs than the non estrous females (p=0.047), however, despite having higher ODUs, the difference between TFMs and estrous females did not reach significance (p=0.308). Additionally, TFMs did not differ from the non estrous females (p=1.00).

For the MeAd, the results were somewhat equivocal; the overall ANOVA revealed a significant effect of genotype (F3, 22=4.002; p=0.02). However, *post hoc* analysis revealed that the only difference in optical density was between the WT males and the estrous females (p=0.015), all other *post hoc* comparisons were non-significant (see figure 46).

Similar equivocal results were obtained for the MeApd, as the overall ANOVA was significant (F3,24=6.225, p=0.003), with the *post hoc* comparisons revealing the WT males had a lower optical density compared to both estrous (p=0.003) and non estrous (p=0.027) females; no other contrasts were significant (see figure 47).

8.5.2 Serum E2 Concentrations

Given the limitations of the assay kit, accurate values were not determined for 10 TFMs and 11 WT males; these values were below 5.97 pg/ml. Thus, we analyzed the serum data two different ways: the <5.97 values were simply taken as equaling 5.97, or these values were removed altogether. Each analysis gave the same conclusion, though as the statistical analyses revealed the TFM affected and WT males did not differ in the amount of serum E2.

When we equate the <5.97 values to 5.97 and include them in the mean and statistical calculations, the one way ANOVA revealed a significant effect of genotype ($F_{2\ 63}=6.945$, p=0.002); *post hoc* analysis revealed that the TFM affected and WT males did not differ

(p=0.882), however, both were lower compared to the ovariectomized estrogen treated females (vs TFMs: p=0.001; vs WT males: p=0.001; see figure 48).

When we removed the 5.97 values, the one way ANOVA again revealed a significant effect of genotype on E2 levels ($F_{2, 42}$=3.843, p=0.029); *post hoc* analysis using the LSD statistic revealed both TFM affected and WT males did not differ from each other (p=0.915), but both were lower compared to the ovariectomized estrogen treated females (vs TFMs: p=0.01; vs WT males: p=0.016).

8.6 Discussion

We report here that male rats carrying the testicular feminization mutation (TFM) displayed increased optical densities (a rough indication of protein levels) in cells of the medial preoptic area (MPOA) and the ventrolateral division of the ventromedial hypothalamus (VMHvl) compared to males. Additionally, the intensity of the ER alpha signal in the VMHvl division was similar in the TFMs compared to both the estrous and non estrous female groups. Together, the data suggest ER alpha immunoreactivity in these regions is feminine in the TFM rat. In cell groups of the medial amygdala, the optical density of ER alpha immunoreactivity was masculinized in both the posterodorsal and anterodorsal divisions. We also observed that the serum E2 values did not differ between TFM affected and WT males.

As mentioned above, TFM affected males display deficits in the performance aspects of sexual behaviour (i.e., intromissions and ejaculations). A previous report indicated systemic injections of 17beta estradiol into gonadectomized TFMs increased the display of intromissions and ejaculations, suggesting ER binding can stimulate sexual behavior in the TFMs (Olsen, 1979). Indeed, radiolabelled E2 binding studies in the

TFMs suggested that the hypothalamic region contained masculine levels of ER binding (Olsen and Whalen, 1982). Paradoxically, though, serum estrogen levels have been reported to be elevated in gonadally intact TFMs, likely due to the conversion of testosterone to estrogen in the medial amygdala (Roselli, Salisbury, and Resko, 1987), and thus it is not clear why TFMs display such mating deficits. However, an examination of radio labeled binding of E2 in the brain is only an indirect measure of ER levels. Additionally, these studies were carried out before the discovery that another estrogen receptor, ER beta, was also expressed within the nervous system. Given that ER alpha and beta bind E2 with similar affinity, the technique used in this previous binding study cannot distinguish the relative levels of these ER species. The current report is a more refined study of the levels of ER alpha, which is important for the normal display of masculine sexual behaviour (Wersinger et al., 1997).

In the current report, the observations of masculine levels of ER alpha within the medial amygdala, but feminine levels within the medial preoptic area, of the TFMs may be useful in explaining why only certain aspects of sexual behaviour are displayed. As stated, Clancy, Zumpe, Michael (1995) reported that increased levels of ER in the MPOA of fadrozole treated males resulted in a decrease in the display of ejaculation; however, application of E2 directly in the medial amygdala stimulated mounting behaviour in males receiving systemic fadrozole treatment (Huddleston, Michael, Zumpe, Clancy, 2003). Thus, the feminine levels of ER alpha within the MPOA of TFMs may be regulating the decreased display of ejaculations, but normal amounts of ER, and hence E2 signaling, in the MeA may be regulating the display of mounting behaviour. It appears, then, that in order for E2 to stimulate the proper mating sequence, levels of ER alpha

levels must be appropriately expressed in regions of the rat brain important for this behaviour. However, evidence also suggests that the androgen receptor may also play a role in stimulation of masculine mating behaviour. Gréco, Edwards, Michael, Clancy (1996) observed colocalization of activated cells (as indexed by Fos immunoreactivity) with androgen receptors in males that mated to ejaculation. McGinnis, Montana, Lumia (2002) reported that direct injection of the anti-androgen, hydroxyflutamide, into the medial preoptic area of male rats maintained on androgen capsules resulted in a suppression of mating activity compared to controls. Interestingly, hydroxyflutamide was most effective at blocking sexual behaviour when applied to the ventromedial hypothalamus (McGinnis, Williams, Lumia, 1996). Thus, both receptor types appear to be important mediators of male sexual activity, and the lack of ejaculatory behavior observed in the TFMs may also be due to the inability of these mutants to respond to testosterone.

Elevated ER alpha levels have previously been observed in the hypothalamus of male rats which have mated several times to sexual satiety; interestingly, satiety is a period of time which corresponds to a complete lack of copulatory behaviour (Phillips-Farfán, Lemus, Fernández-Guasti, 2007). ER alpha immunoreactivity has also been observed to be elevated in the MePD and MeAD of male rats which do not display any components of masculine sexual behaviour (termed non-copulators; Portillo, Díaz, Cabrera, Fernández-Guasti, Paredes, 2006). While we did not observe significantly different levels of ER alpha intensity in both of these regions of the amygdala, the optical density of the TFMs was consistently elevated compared to the WT males. It has also been reported that AR immunoreactivity is decreased in the medial preoptic area and

ventromedial hypothalamus in male rats that have mated to sexual satiety (Fernandez-Guasti, Swaab, Rodríguez-Manzo, 2003); this effect of mating on AR expression is independent of serum testosterone levels as there were no differences in the sated males compared to the controls (Fernandez-Guasti, Swaab, Rodríguez-Manzo, 2003). Because the ligand bound androgen receptor positively regulates the transcription and translation of androgen receptor protein levels (Handa, Kerr, DonCarlos, McGivern, Hejna, 1996), and given that TFMs are insensitive to the actions of androgens, the current orthodoxy predicts that AR levels would also be low in the brain of these mutants (however, this has never been directly examined). Thus, given results from past research (mentioned above), the relative levels of the androgen and estrogen receptors in the TFM brain appear to mimic that of the sexually sated rat. What is more, this androgen/estrogen receptor profile would also be predicted for castrated male rats (i.e., decreased AR and increased ER alpha). Thus, if we consider the evidence as a whole, the testicular feminization mutation seems to affect not only the androgen receptor system, but indirectly affects the expression of the ER alpha isoform, as well.

The expression of the estrogen receptor protein appears to be under negative feedback regulation from E2; removal of E2 would thus result in elevated levels of ER alpha in select areas of the nervous system. In all the areas examined in the current report, TFMs displayed higher average optical densities compared to the WT males (although, significance was only reached in the MPOA and VMH), which paradoxically suggests that the TFMs would have lower serum estrogen levels. However, we observed serum E2 levels to be equivalent in the TFMs and WT males. One other study reported that E2 levels were approximately two fold higher in the TFMs compared to WT males

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(Roselli, Salisbury, Resko, 1987). Nevertheless, the levels of serum E2 in the TFMs suggest that ER alpha levels should be decreased to masculine levels. That we observed equivalent E2 serum levels in the TFMs and WT males, but lower intensities in ER alpha staining in the hypothalamus of WT males, may suggest that negative feedback of ER alpha expression is lost in the TFMs in both the medial preoptic area and ventromedial hypothalamus. We can only speculate as to why this may be the case, however, androgen receptor activation, during a critical developmental window, may be necessary. This contention is supported by a study in which blockade of androgen receptors in male rats immediately after birth resulted in feminine levels of ER alpha (i.e., higher) expression in the MPOA, whereas androgen treatment of female rats resulted in masculine levels of ER alpha (i.e., decreased) expression (Kühnemann, Brown, Hochberg, and MacLusky, 1995). The effects of androgens on ER alpha levels appear to be limited to a critical developmental window that encompasses the first 10 days of life (Kühnemann, Brown, Hochberg, and MacLusky, 1995). Thus, the data in the current report suggest that the elevated levels of ER alpha observed in the MPOA and VMHvl of TFMs may have resulted from a lack of androgen stimulation developmentally.

However, an alternative possibility is that conversion of testosterone to estrogen *within* aromatase expressing cells is the critical factor necessary in lowering ER alpha levels, and that serum levels of estrogen are not as important despite that fact that 17 beta estradiol is lipophilic and can gain access to cytoplasmically located ER alpha. We suggest this possibility based on the findings of Roselli, Salisbury and Resko (1987), in which it was observed that aromatase activity was normal in the medial amygdala of adult TFM affected males, but was severely reduced in the medial preoptic area.

In the current report, we use optical densities as a rough indicator of ER alpha protein levels; the use of this technique is similar to in situ hybridization for mRNA, and is similar in principle to western blotting, which uses antibodies to reveal protein expression levels. Ovariectomy has been reported to result in an increase in ER alpha mRNA (Lauber, Mobbs, Muramatsu, Pfaff, 1991) and protein (Weiland, Orikasa, Hayashi, McEwen, 1997) in the ventromedial hypothalamus, as well as mRNA content in the medial amygdala (Lauber, Mobbs, Muramatsu, Pfaff, 1991), with a subsequent reduction in mRNA and protein content following estrogen treatment. Our results are consistent with these previous reports as we observed estrogen treatment to decrease the intensity of the ER alpha signal in the VMHvl of the estrous females compared to the non estrous females, suggesting the analysis of optical densities in immunocytochemically stained tissue may be an acceptable (rough) indicator of protein expression levels. As we were able to directly analyze cells containing ER alpha immunoreactivity, we achieved greater spatial resolution compared to the use of Western blotting. It has previously been observed that the preoptic area does not contain a sex difference in terms of staining intensity, however, a sex difference has previously been observed in the ventromedial hypothalamus in which males displayed slightly lower intensities (Lauber, Mobbs, Muramatsu, Pfaff, 1991). Inconsistent with this previous study, we did not replicate either of these results as we did find a sex difference in staining intensity in the MPOA, in which males displayed decreased optical densities compared to the estrous females. Additionally, there were no differences detected in staining intensity in the VMHvl between WT males and estrous females in the current report. However, the hormone regime in that previous study was equivalent in both male and female subjects (animals

were gonadectomized and given equal doses of estrogen), whereas in the current study, males were left gonadally intact while females were gonadectomized and given estrogen implants that were in the pharmacological range. Thus the disparity in results from previous research with the current study could easily be attributed to differences in the levels of circulating estrogens.

8.7   Summary and Conclusions

In summary, we have observed increased staining intensity of ER alpha immunoreactivity in the medial preoptic area and ventromedial hypothalamus (ventrolateral division) of TFMs compared to WT males. Given what has been reported in the literature regarding the levels of ER alpha immunoreactivity in the normally mating male rat, we suggest that the differential ER alpha levels observed in the TFMs compared to the WT males may have consequences for the proper display of masculine sexual behaviour.

Figure 43 Figure 1. Average ER alpha intensity (ROD) in A) the medial preoptic area (MPOA), B) the ventrolateral division of the ventromedial hypothalamus (VMHvl), C) the posterodorsal division of the medial amygdala (MePD), and D) the anterodorsal division of the medial amygdala (MeAD). Note: A) *= WT male<TFM (p=0.009); WT male< EB ovx female (p=0.007); WT male<Non estrous female (p<0.001). B) *= WT male <TFM (p=0.02); **= WT male< Non estrous female (p= 0.001); ***= Non estrous female> EB ovx female (p= 0.047). C) *= WT male< EB ovx female (p=0.015). D) *= WT male< EB ovx female (p= 0.003); **= WT male< Non estrous female (p=0.027).
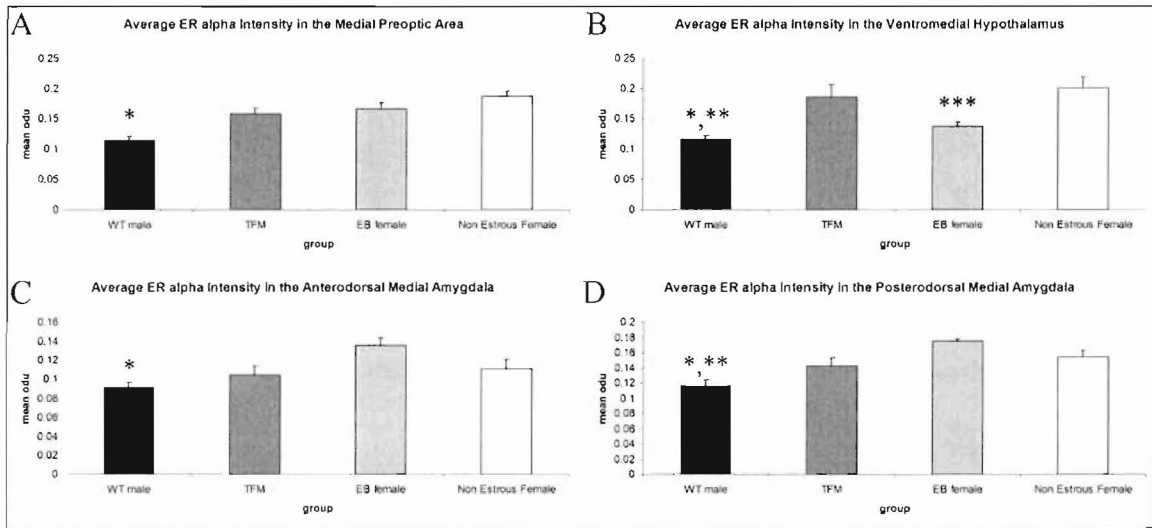

Figure 44 ER alpha immunoreactivity in the medial preoptic area (MPOA) of WT male, TFM, Estrous and Non Estrous females.
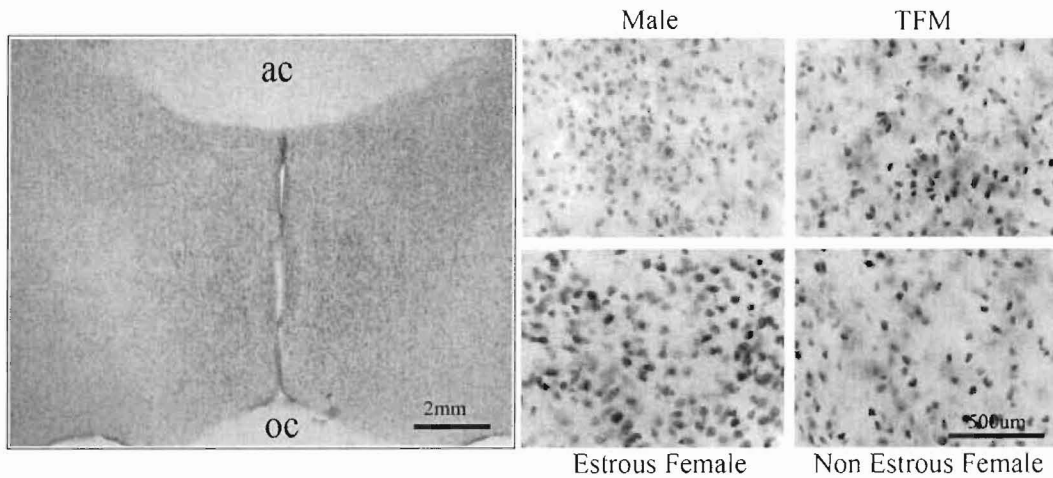
Figure 45 ER alpha immunoreactivity in the ventrolateral division of the ventromedial hypothalamus of WT male, TFM, Estrous and Non Estrous females.

Figure 46 ER alpha immunoreactivity in the anterodorsal division of the medial amygdala of WT male, TFM, Estrous and Non Estrous females.
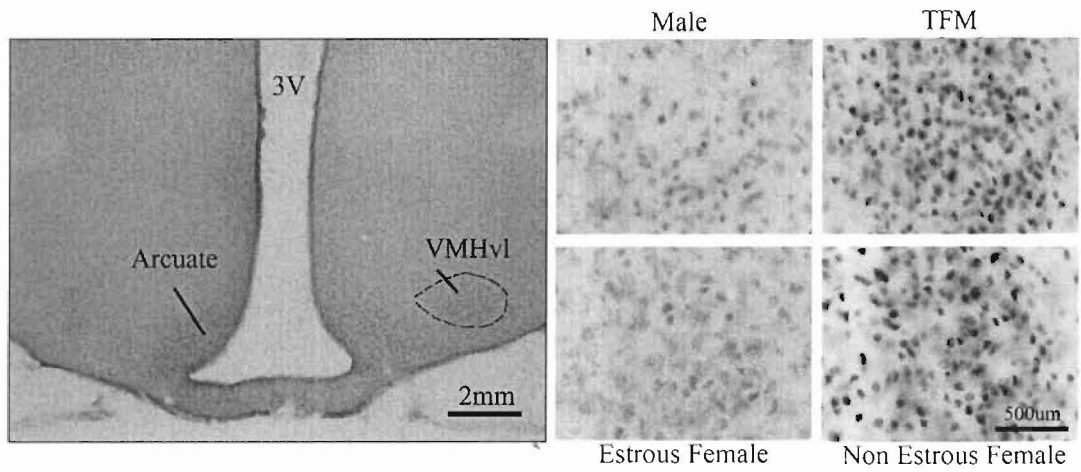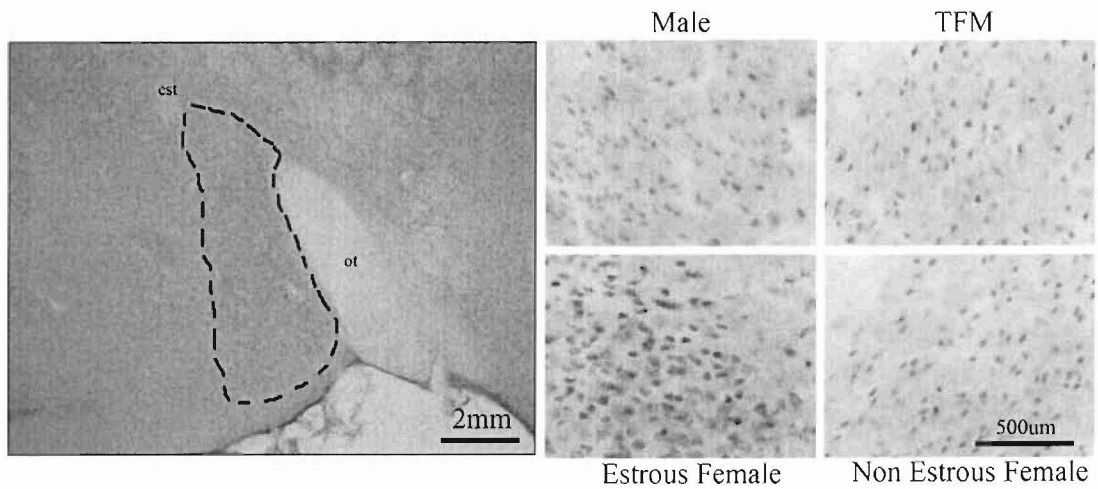
Figure 47 ER alpha immunoreactivity in the posterodorsal division of the medial amygdala of WT male, TFM, Estrous and Non Estrous females.
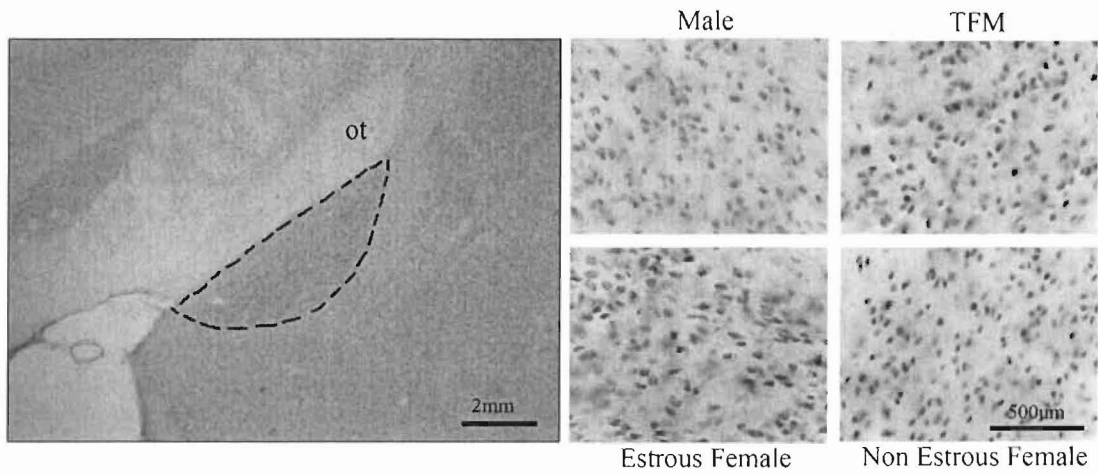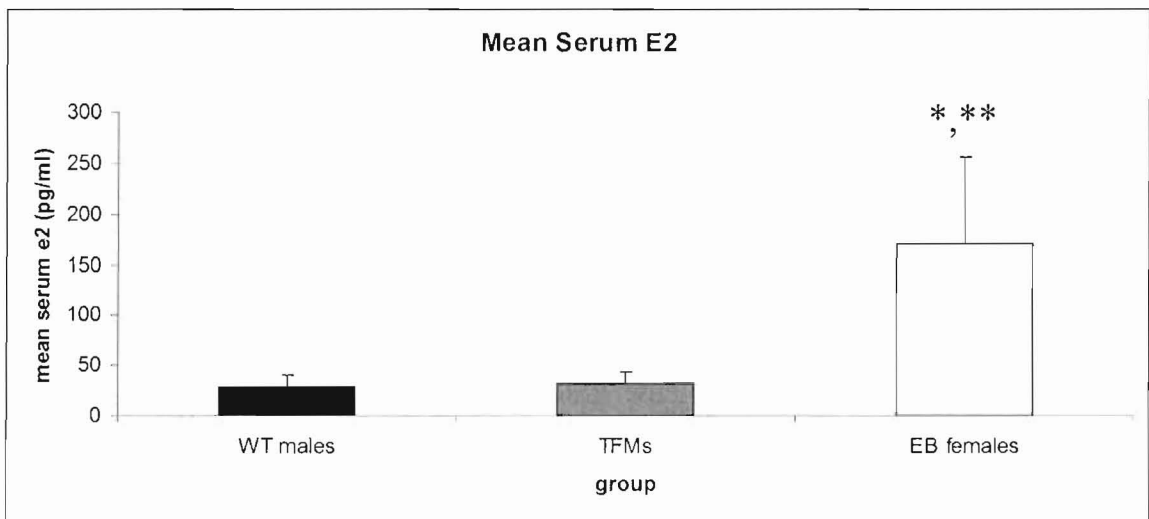

Figure 48 Mean Serum E2 concentrations in WT males, TFMs, and Estrous Females. Note: *= WT males< Estrous females (p=0.001); **= TFMs< Estrous females (p=0.001).

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CHAPTER 9 GENERAL CONCLUSIONS CHAPTER

9.1   General Discussion and Concluding Remarks

The experiments carried out in this dissertation have provided a more general idea of the

behavioural and physiological deficits as a result of the androgen receptor mutation. The

initial studies examining the sexual behavioral deficits of the TFM animals were mostly

incomplete or did not give an accurate picture of the nature of the deficits due to

methodological issues (i.e., Olsen, 1979; Shapiro, Levine, Alder, 1980). Additionally,

interesting observations, such as the ability of some of the TFMs to display the full

ejaculation-like response despite the lack of a penis, were not addressed in any manner.

In the first set of experiments presented in this dissertation (chapter 2), the TFMs were

observed to display very similar sexual behaviour deficits as that observed in Shapiro,

Levine, and Adler (1980), however, the second cohort of TFMs that were analyzed in the

current dissertation (i.e., from chapter 4: Partner preference in the TFMs) was more in

line with the observations made in Beach and Buehler (1977). Together, these studies

highlight the high degree of variability these animals display in terms of sexual

performance, despite that all the animals have the exact same mutation. We can also

conclude that the mutation does not affect the ability of the TFMs to become sexually

aroused (as they displayed normal mount latencies), and preferred to spend time with a

sexually receptive estrous female (Chapter 4; Partner preference in the TFMs).

Subjectively, however, it appeared the TFMs did not perform sexually during the last

three tests of sexual activity in the standard mating arena; performance was also very

poor in the bi level chamber. One central issue that is still left unexplored is reward in the TFMs. It is has been observed that penile stimulation and ejaculation are quite rewarding (sexually experienced males will bar press for access to estrous females), and thus sexual experience serves to ensure that the behaviour will be repeated in the future. Thus, the lack of sexual performance in the bi level chamber may have been due to a lack of reward; this, however, is tempered by the observation that a majority of animals did not make an attempt to pursue the estrous females. Interestingly, dopamine has been observed to play a role in many behaviours that are rewarding and that may become addictive (such as gambling, taking drugs, smoking, etc), additionally, evidence has been reported that shows androgens, such as testosterone, play a role in the expression of tyrosine hydroxylase, the terminal enzyme in dopamine production. Thus, the TFM could potentially be an interesting model to study reward (or lack thereof, termed anhedonia). Dopamine also plays a role in the stimulation of sexual behaviour (reviewed in Hull and Dominguez, 2007); reports indicate DA is released in the medial preoptic area and nucleus accumbens just before and during sexual behaviour. Thus, it is interesting to speculate that part of the deficit displayed by the TFMs may be due to deficient DA production or release.

We also examined more closely how the androgen receptor mutation can affect the structure and functioning of the central nervous system. Beach and Buehler (1977) initially predicted that the circuitry regulating male sexual behaviour was masculine, but the ability of these areas to become activated was compromised due to the androgen receptor mutation. This was quite a bold statement given the state of the field of behavioral endocrinology at the time. Surprisingly, very little was done to actually

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confirm or reject this hypothesis until examinations of the morphology of the nervous system was more closely examined (by the Breedlove lab and the studies in the current dissertation). It was observed in the current dissertation that cells within the medial preoptic area, the most important region important for mating, were feminine in size, however, this area, as well as the medial amygdala, were essentially activated normally (although the medial amygdala displayed more Fos immunoreactivity). Thus, it appears the androgen receptor mutation does not negatively impact the ability of these regions to become stimulated normally. However, it appeared that the accessory olfactory bulb in the TFMs displayed decreased 'output' (or increased GABAergic tone) as Fos-ir was observed to be different compared to the WT males; thus the androgen receptor may be necessary for proper function of the AOB. Additionally, this observation merits further attention as it may be the case that the TFMs display subtle behavioural differences that are not detected by the arousal tests used in the current dissertation. Additionally, these observations complement other studies showing that parts of the TFM nervous system are not fully masculine; thus deficits maybe due to incomplete masculinization of the nervous system. To date, no studies have examined the development of the TFM nervous system; however, this promises to be a rich area of study.

Another interesting aspect about arousal regards the use of 50 kHz ultrasonic vocalizations (USVs) by male rats and mice to enhance sexual receptivity in the estrous females. It has often been observed in the lab that pairing sexually receptive females with TFMs results in several confrontational displays by the estrous female when a TFM attempts to mount. Thus, the decreased emission of 50 kHz USVs observed from the TFMs (e.g., chapter 6) complements these observations, and leads us to speculate that the

females do not necessarily recognize the TFMs as viable mating partners. The experiments in this chapter also highlight the importance of the need to consider how a mutation in one animal can affect the behaviour of a partner. The experiments in this chapter complimented previous studies that have also shown the androgen receptor has a role in the production of 50 kHz ultrasonic vocalizations, but in a different way as it allowed us to confirm the role of the androgen receptor directly. Observations of decreased USV production lead to the examination of a protein, Foxp2, important for these calls (i.e., chapter 7). This study observed a sex difference in the expression of this protein in an area thought to be important for USVs, the cerebellum, in mice and rats, but also in the acquisition and production of speech in humans.

Interestingly, previous observations suggested the TFMs may be under stress, and this could have possibly negatively affected sexual performance. We can confirm in the current dissertation that this does not appear to be the case as the TFMs did not display increased anxiety in the elevated plus maze. This observation compliments the study of TFM spatial ability in the Morris Water Maze (Jones and Watson, 2005); female rats often display 'thigmotaxis' in the Morris Water Maze (a behaviour in which female rats swim in circles in the Morris Water Maze, and this is thought to be a sign of distress) however, males do not display this behaviour. Additionally, the TFMs also do not display thigmotaxis, and thus despite the high levels of corticosterone that have been observed in the TFMs, this does not seem to translate into more anxiety behaviour.

As stated, Oslen (1979) suggested that the lack of sexual performance was due to a lack of aromatization of testosterone to estrogen. However, these observations did not fit with previous reports suggesting that estrogen production was high and that the

hypothalamus contained estrogen binding sites (Olsen and Whalen, 1982; Rosselli, Salisbury, and Resko, 1987). Upon closer examination, it was observed in the current dissertation, that the levels of estrogen receptor alpha were feminized in the TFMs. This may suggest that the lack of a proper display of sexual behaviour by the TFMs may be due to improper masculinization during development, as androgen receptor activation appears to be necessary for this process. These results cast further doubt on the conclusions of Olsen (1979); additionally, this previous study tested the effect of androgens and estrogens on sexually naïve animals and did not counterbalance the hormone regime, and so the data is confounded by order effects. Finally, the sexual abilities of rats increase following repeated pairings with estrous females, further confounding the results of Olsen (1979). A closer examination of the events involving the regulation of the estrogen receptor during development in the TFMs is necessary.

The data in the current dissertation further support the role that the androgen receptor plays in motivated behaviours such as sexual behaviour. The current studies also highlight the necessity of androgen receptor activation in the proper masculinization of the structure and function of the regions supporting this behaviour.

9.2 References

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