THE ROLE OF ANDROGEN RECEPTORS IN SPATIAL LEARNING AND HIPPOCAMPAL MORPHOLOGY: ANDROGEN INSENSITIVE MALE RATS IN THE WATER MAZE

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ABSTRACT

The current literature suggests that the masculinization of the brain occurs in response to the conversion of androgens, such as Testosterone, to estrogens in a process called aromatization. This Aromatization Hypothesis has been supported not only in research looking at reproduction, but also non-reproductive behaviours such as cognition. Recently, however, several challenges to this hypothesis have emerged, specifically in regards to non-reproductive behaviours, prompting a closer examination of the role of both androgen and estrogen receptors in organising the Central Nervous System (CNS) and the resulting behaviours. A novel way of doing this is through study of male rats which are insensitive to androgens. Because of a genetic mutation (the testicular feminization mutation (*tfm*)) in the gene coding for the androgen receptor, these males, despite the presence of testes and circulating testosterone, develop a female phenotype; aromatization, however, should be unaffected, thus providing for a masculinized CNS with regards to cognitive behaviours. In this study, *tfm*-affected males (*tfm*'s) are compared with normal males and normal females in the Morris Water Maze, a task in which males typically perform better than females. The hippocampi (HPC) were then removed and the volume of this structure and several areas was estimated. A male superiority was found in the task, while *tfm*'s initially seemed to exhibit an intermediate level of performance, showing a female-type performance early in the testing trials, but achieving a male-typical performance well before the females. Furthermore, there appeared to be several areas of the HPC in which *tfm*'s showed enlarged volumes relative to both males and females, especially in the dentate gyrus. This further challenges the aromatization hypothesis, and suggests further examination of the role of AR and ER in both the control of spatial behaviours, and the hippocampal morphology.

DEDICATION

For getting me through this far, I must thank

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TABLE OF CONTENTS

Approval ii

Abstract iii

Dedication iv

Acknowledgements v

Table of Contents vi

List of Tables viii

List of Figures viii

INTRODUCTION 1

COGNITIVE STUDIES 2

Morris Water Maze 2
Radial Arm Maze 3

SEX DIFFERENCES IN SPATIAL ABILITY 4

Hormonal Influences On Cognition 6
Organizational Effects 6
Activational Effects 9

NEURAL SUBSTRATES UNDERLYING SPATIAL-NAVIGATIONAL ABILITIES 11

The Hippocampal Formation 11
Lesion Studies 13
The CA3 Field 13
Dentate Gyrus 13
The CA1 Field 14
Subicular Complex And The Entorhinal Cortex 14
Gonadal Hormones and the Hippocampus 15
Hormonal Control Of Sexual Dimorphisms In The Hippocampus 15
Localisaton Of AR & ER In The Hippocampus 17
ER Localisaton 17
AR Localisaton 18
Physiological Effects of Gonadal Hormones in the Hippocampus 18
Estrogen 18
Androgen 20

Sexual Differentiation **22**
Peripheral Differentiation 22
Central Differentiation 23
Testicular Feminization Mutation 23
PROPOSAL **25**
METHODS **26**
Animals 26
Handling 26
Testing Room and Apparatus 27
Water Maze Protocol 27
Identification of Females and *Tfm*'s 28
Histology 28
Water Maze Analyses 28
Morphological Analyses 29
RESULTS **32**
Morris Water Maze 32
Escape Latency 32
Distance to Platform 32
Time spent in the Rings 33
Time spent in the Quadrants 33
Hippocampal Morphology 38
DISCUSSION **43**
Morris Water Maze 43
Hippocampus 44
GENERAL DISCUSSION **46**
List of Abbreviations **49**
References **52**

LIST OF TABLES

Table 1 Punnett Square detailing the genotype of offspring of males and carriers 24
Table 2 Area Volume Measurements 41
Table 3 Differences in Hippocampal Volume 42

LIST OF FIGURES

Figure 1 Rat Hippocampus 11
Figure 2: Dorsal Hippocampus (Stereotaxic) 30
Figure 3: Ventral Hippocampus (Stereotaxic) 31
Figure 4: Latency to Platform 35
Figure 5: Latency to Platform: Day Three 36
Figure 6: Latency to Platform: Day Four 37
Figure 7: Latency to Platform: Day Five 38
Figure 8: Dorsal Hippocampus visualized with Thionin 40

INTRODUCTION

Since the early 1900's, sex differences in cognitive abilities have been examined in rats (McNemar & Stone, 1932), and have been used to model the known differences in humans. While a wide range of sexually dimorphic cognitive abilities has been noted in humans over past decades (Kimura, 2002), to understand the molecular and cellular mechanisms by which these differences are organized, we must necessarily use animal models. Thirty years of research, focusing on rodents such as rats and meadow voles, have greatly increased our understanding of the organization of these dimorphic abilities. Early research showed that the gonadal hormone testosterone was capable of creating these differences (Stewart et. al., 1975; Joseph et. al., 1978). However, the formulation of the aromatase hypothesis (Naftolin et. al., 1975) whereby the activities of testosterone in masculinising the brain were shown to be due to the conversion of that hormone to the estrogen, 17-β estradiol, suggested that spatial abilities may be organized through the activity of estrogen receptors as opposed to the androgen receptor. While this was initially confirmed by Williams and colleagues (1990), more recent evidence (Isgor & Sengelaub, 1998; 2003) would suggest that there is a role for the androgen receptor as well in this regard.

The purpose of these studies is to determine the extent to which the androgen receptor is responsible for the organization of spatial behaviour, as opposed to the known effects of the estrogen receptor.

COGNITIVE STUDIES

While the study of gender differences is often related to reproductive behaviours, non-reproductive behaviours have also been extensively studied. Specifically, cognitive differences between the genders have been discussed since at least the early 1930's (McNemar & Stone, 1932). The advent of animal testing in psychology with the likes of investigators such as Thorndike, and later Lashley, relied on mazes which were essentially open fields with the addition of extra walls. These mazes, such as the Lashley III maze or the Hebb-Williams maze typically elicited a male superiority in navigational ability, though the interpretations were often tempered by methodological issues that were not confronted by these early researchers (Beatty, 1979). Yet despite these early problems, there was a consistently described male superiority (e.g. reduced error rates) in these and other mazes, when comparing species such as rats or meadow voles (Williams, Barnett & Meck, 1990).

Morris Water Maze

In 1981 a new procedure for studying navigational ability was pioneered by Richard Morris, and has since become one of the most used techniques in the study of spatial and navigational abilities. It is a circular tub, filled with water. The sides of the tub are white, offering few intramaze cues. The water is made opaque through the addition of some non-toxic additive (Morris initially used fresh milk), and a platform is placed at a certain location within the pool. The initial use of this procedure compared rats who could escape by finding either a visible platform (e.g. slightly elevated above the water's surface), or an invisible one (slightly below the surface of the water), both of which remained in a fixed position (Morris, 1981). It was found that while the rats seeking a visible platform escaped much quicker than those trying to find the non-visible one, the latter group certainly exhibited strong learning as determined by a constantly decreasing latency to find the platform. This particular finding was interpreted as revealing the use of spatial cues in finding the fixed, non-visible platform, and thus, subsequent tests examining spatial-navigational abilities have capitalized on this fact.

While this maze, which has come to be known as the Morris Water Maze (MWM) is relatively simple in comparison to the early navigational tests, it is difficult enough to require good navigational abilities to solve (Brandeis, Brandys & Yehuda, 1989). Furthermore, the use of a good tracking system will allow for a number of different analyses, including the distance travelled to the platform, the escape latency, and the amount of time spent in various areas of the pool.

Radial Arm Maze

Another excellent test for examining spatial/navigational abilities is the Radial Arm Maze (RAM). First pioneered by Olton and Samuelson in 1976, this maze features a central hub from which a number (typically 8 or 12) of different arms protrude (Williams, Barnett & Meck, 1990). A predetermined pattern of arms is baited with food, to try to get the animal to investigate the entire length of these correct arms. Any entrances into an unbaited arm (past a specific point) are considered to be an error. Analyses can examine the number of arms entered (choices) until all food is removed, the number of errors, and the number of choices until an error. This test is often used as it is less sensitive than previous mazes to the increased locomotor activity of females; however, one of the problems with this protocol is the question of motivation. A standard, laboratory housed rat receives food *ad libitum*, and thus, hunger is rarely an issue. So, animals used in the RAM are typically food deprived such that they are maintained at a weight that is somewhere between 80-90% of their pre-experimental body weight. The MWM is nicer in the sense that rats, while being naturally born swimmers, are intrinsically motivated to exit the maze. However, the RAM has been used to examine different aspects of memory. While the MWM is typically used to examine only reference, or long-term memory, a RAM protocol can be organized to examine both reference and working (or short-term) memory (Williams, Barnett & Meck, 1990).

SEX DIFFERENCES IN SPATIAL ABILITY

Like their predecessors, both the MWM and the RAM are sensitive enough to find small but reliable sex differences. This has not been without some controversy, as a number of studies have failed to find a difference on both of these tasks, so a detailed analysis of the findings is warranted.

The literature reveals that, particularly with respect to the MWM, a number of different protocols have been used, many of which have resulted in a sex difference. Roof, for example, has used a number of testing procedures. Twice (Roof, 1993a; Roof & Havens, 1992), rats were tested once a day for six days, with control males outperforming control females. Later, Roof and Stein (1999) used several protocols in which rats were tested twice a day for ten days, and again, differences were found when the release positions in trial 1 and 2 differed on each day of testing. In contrast, Isgor and Sengelaub (1998) subjected rats to their MWM six times a day, for seven days and still found a difference. Similarly, Frye (1995) found a sex difference when testing was done six times a day, for two days, though those two days were not sequential.

Reports unable to find a sex difference also exist (Warren & Juraska, 1997; Bucci, Chiba & Gallagher, 1995; Healy, Braham & Braithwaite, 1999), and again, several different protocols exist. Warren and Juraska, for example, carried out 16 trials all in one day, whereas both Bucci et al. (1995) and Healy et al. (1999) used several trials a day, over several days.

A closer look at the protocols used in each of these studies, however, reveals a difference in the pre-experimental handling of the animals. All of the studies mentioned above that were unable to find a sex difference pre-trained their animals in a water maze prior to experimental testing and analysis; by comparison, none of the studies that found a sex difference performed this particular procedure, with the exception of Isgor and Sengelaub (1998). Unfortunately, none of the authors noted the performance that occurred during the pretraining.

One theory that has been used to explain this discrepancy is suggested by Cain, Hampson & Boon (2003). Specifically, the suggestion is that the testing procedure causes a differential stress reaction in females that prevents them from acquiring the

task as quickly as males. Often, this is seen in the increased thigmotaxis displayed in females, by comparison with males. Thigmotaxis manifests itself behaviourally as floating in water, and swimming along the outer edge of the pool, closest to the pool walls. It is possible that the introduction of naïve animals to the MWM causes increased stress in females that prevents them from acquiring the task as quickly as males, thus the lack of difference in those studies in which analysis commences following pre-training. But this does not explain the seemingly contradictory finding from Isgor and Sengelaub (1998, 2003). Those studies did use a pretraining regimen prior to experimental testing, and yet, a sex difference was clearly apparent. An analysis of the protocols does not suggest that there was any major difference in the pretraining methodology employed by the varying investigators. Isgor and Sengelaub (1998) used two trials, one with and one without the (submerged) platform, while both Warren and Juraska (1997) and Bucci et al. (1995) used three trials with a visible platform in the former case, and a submerged one in the latter. The striking difference comes in the size of the pool used by Isgor and Sengelaub (1998); they used a tub that was 2.75 metres (m) in diameter. In comparison, the largest tub used by any other group was 2.00 m (Frye, 1995) and 1.95 m (Healy et al., 1999). It seems likely that the size of the pool employed by Isgor and Sengelaub was large enough to detect a sex difference even after females had already been acclimated to the physical stresses of a water maze and the type of task they were intended to solve. Previous work (Perrot-Sinal, et al., 1996) has shown that exposure to the MWM prior to experimental testing will result in an eradication of the sex difference, but again, the difficulty of the task in this study (1.50 m diameter pool) was likely less imposing than that of the Isgor and Sengelaub experiment. Also, Perrot-Sinal et al. (1996) gave their rats twelve training swims – four trials a day for three days. Thus, the combination of increased training and decreased difficulty likely explains the divergent results.

The RAM has also been somewhat contentious as a tool for measuring sex differences; there have been a number of studies that have failed to find a difference in their protocols. Both van Haaren, et al. (1987) and Juraska and colleagues (1984) reported no sex difference, whereas Williams, Barnett & Meck (1990) found the opposite. Again, the difference may lie in the difficulty of these groups' respective

protocols. The two former groups had baited all of the arms of the maze with food, scoring both the number of correct choices and either the number of errors or the total number of choices within the time allotted. However, in the Williams et al. (1990) study, a specific pattern of baiting was utilized. These researchers only baited 8 of the 12 arms, and the animals were required to find the food pellets at the end of the correct arms. They were then scored on several measures, including the median number of choices required to find all of the food. Again, it seems possible that the task is made more difficult in this protocol and thus sensitive enough to detect sex differences in spatial/navigational abilities. Taken together, data from these two paradigms suggest that there is a genuine difference in spatial ability favouring males, in agreement with the human literature (Hampson 1995; Kimura 2002).

Hormonal Influences On Cognition

As is often the case, researchers interested in sex differences in behaviour have looked first to the gonadal hormones, to see if these are they are producing or influencing these differences in any way. Not surprisingly, it is generally accepted that male superiority on spatial tasks is related to the increased production of testosterone both *in utero* and shortly after birth. These organizational effects are now known to be sufficient to alter spatial learning in adulthood, however, activational effects - the response of the Central Nervous System (CNS) and the resultant behaviour to naturally occurring fluctuations in hormone titers - have also been found to contribute to differences in maze solving ability (Kimura, 2002).

Organizational Effects

Due to reports of sexual dimorphisms in non-reproductive behaviours, Stewart et. al. (1975) examined the hormonal control of spatial learning through the administration of androgens to females. They discovered, using a Lashley-III maze, that males performed better on this task (as measured by error rate and trial to criterion), but when females had been injected with testosterone propionate (TP) on the two days following birth, they performed similarly to males, and better than control wild type (WT) females.

Following this, Joseph et al. (1978) noted a similar result, and also finding that males treated neonatally with cyproterone acetate (a steroidal anti-androgen) and neonatally castrated males performed similarly to normal females (i.e. worse than control males). Furthermore, the authors noted that this was an organizational effect; gonadectomy in adulthood did not alter performance within each sex, while both male groups (castrate and control) performed better than both female groups.

Roof and Havens further sought to elucidate this mechanism (1992). However, their study examined the effect of neonatal TP on non-castrated males as well as females. Using the MWM, they noted, similarly to Stewart et al., that control males performed equally well as TP-injected females, and both were better than control females. Intriguingly, though, they noted that intact, TP-injected males performed slightly worse than control males, requiring more trials to reach an asymptote in performance. To further examine this effect, Roof (1993a) examined control or neonatally TP-treated (either 150- or 300μg) males and (75- or 150µg) females on both the MWM and the RAM. Both experiments yielded similar results in that there was an interaction between sex and TP level. In males, controls performed better than the 150μg group, which in turn performed better than the 300μg group. In females, the opposite was true; the more neonatal TP, the better the performance. This suggests that there is an optimal level of testosterone required for spatial performance.

As will be discussed below, testosterone's effects in the CNS of mammals is known to be mediated, at least partially, by the actions of its estrogenic metabolite, 17-β estradiol, acting on its cognate receptor. Williams, Barnett and Meck (1990) sought to determine whether or not estradiol has any effect on the performance of females in the RAM. Males were either castrated or sham-operated neonatally, and compared to females injected neonatally with either estradiol or an oil vehicle. As before, control males were found to perform better on the 12-arm version of the RAM, making fewer errors and requiring fewer choices to criterion than the control females and the castrated males. The estradiol-treated females performed similarly to control males and better than the other two groups, suggesting that the effects of testosterone may be partially due to the actions of its estrogenic metabolite. Furthermore, all animals were castrated at 45 days, prior to puberty, to rule out any possible activational effects (Williams et al.,

1990). To determine if testosterone was acting as an estrogen, Williams and Meck report (1991) on a study examining males injected neonatally with the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD). In this study, control males performed better on the 12 arm RAM than the ATD group, suggesting that the activity of testosterone may be mediated by the ER. Unfortunately, there was no female comparison group. Also, the patterns of scores on this test were somewhat different than those seen in an identical protocol (Williams et al., 1990). While the possibility does exist that testosterone (T) acts as an estrogen via the estrogen receptor (ER) to masculinize spatial behaviour, a more direct comparison does need to be made. One further complication comes from the finding that ATD can prevent T binding to the androgen receptor (Kaplan & McGinnis, 1989), suggesting that this protocol may have inhibited AR function, as well. However, these results do provide convergent evidence for the support of the aromatization hypothesis (Naftolin et al.,1975). Again, this hypothesis posits that the masculinization of the brain results from the conversion of androgens, like T, to estrogens, and that it is the activity of these hormones, acting through the ER, that differentiate the brain along sexually dimorphic lines.

Isgor and Sengelaub attempted to examine this question through the neonatal administration of the anti-androgen flutamide (flu) to males, comparing them to controls, males castrated in adulthood, control females, and females injected neonatally with TP, estrogen benzoate (EB) or the non-aromatisable androgen, dihydrotestosterone propionate (DHTP) (1998; 2003). As expected, control males performed better than the control females. They also reported that both TP and DHTP injected females performed as well as males, and that the flu treated males performed similarly to the control and EB-treated females. The performance of the EB-treated females is somewhat surprising, given the findings from Warren and Meck (1990). However, it does agree well with the finding from Joseph and colleagues (1978) that the administration of cyproterone does result in a female typical pattern of behaviour. These results, then, suggest that circulating perinatal testosterone may function to organize male-typical behaviour by acting as an androgen *per se,* and serve as a challenge to the aromatization hypothesis, at least in regards to spatial memory.

Activational Effects

Activational effects can be described as transient changes in physiology and/or behaviour resulting from exposure to gonadal hormones in adulthood. Once a neural population has been organized (which is a permanent effect) by gonadal steroids, it may be further acted upon by these hormones in adulthood. As described above, the differences seen in spatial behaviour can be completely accounted for by the organizational effects of gonadal hormones. This is not to say, however, that circulating levels of T or 17β-estradiol (E2) can not have an effect in adulthood. Many of the studies looking to understand this effect have utilized the estrous cycle in female rats to explore the effect of gonadal secretion on spatial behaviour.

The effects of these experiments have been less equivocal. The general findings are that females perform more poorly on tasks of spatial memory (MWM or RAM) during the period of pro-estrus (behavioural estrus) when estradiol levels are at their highest (Frye, 1995; Warren & Juraska, 1997). This agrees well with evidence from Galea et al. (1995), showing that female meadow voles with high estradiol levels performed more poorly than both males and low-E2 females. However, there is an increase in hippocampal spine density in the CA1 field of the hippocampus on the day of pro-estrus (Woolley & McEwen, 1992), which appears to be correlated to the decrease in performance. Chesler & Juraska (2000) have suggested that it may be the effects of progesterone (P) that are interacting with estrogen to produce these effects. They noted that injections of both E2 and P, but not either alone, significantly inhibits learning in the MWM. As P levels are also at their highest during the day of pro-estrus, it may be that the presence of P is attenuating the performance of these females at this time.

Less work has been done with regards to the activational effects of hormones in males. One group, however, has noted that injection of flu, the androgen receptor (AR) antagonist, in adulthood does decrease MWM performance (Naghdi et al., 2001). This group also discovered that the non-monotonic effects of testosterone due to dose also exist within adulthood, as males given high levels of TP (40 & 80 µg) showed poorer MWM performance than males. This again suggests that there are activational effects in adult males, and due to the effects of flu, these are likely mediated by the activity of the

AR. However, more work clearly needs to be done to examine this effect in greater detail.

NEURAL SUBSTRATES

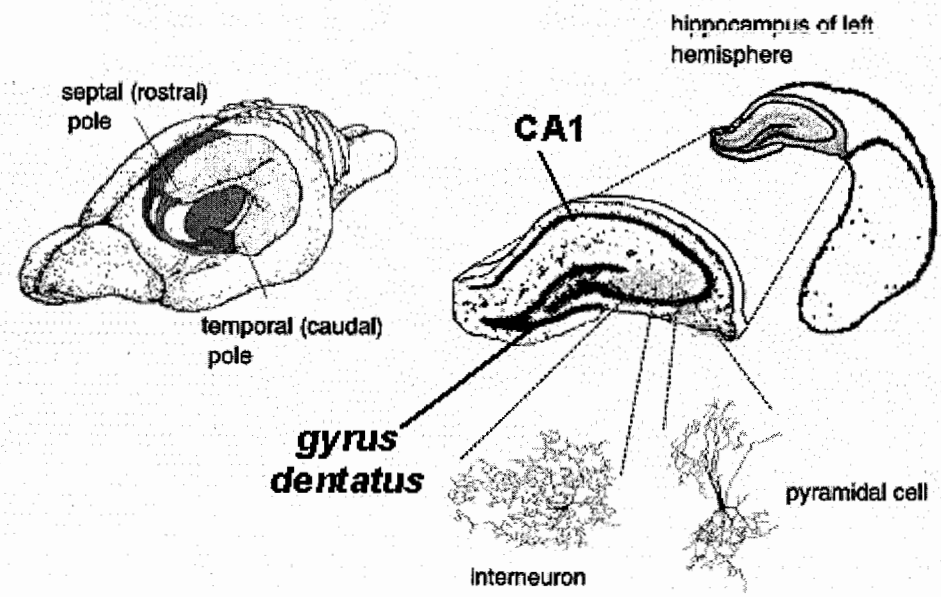
UNDERLYING SPATIAL-NAVIGATIONAL ABILITIES

A number of cortical and subcortical areas have been proposed to affect spatial ability in the rat, including the medial frontal cortex (Kolb & Cioe, 1996), the septum (Ikonen et. al., 2002), the amygdala (Packard & Teather, 1998) and the mammillary bodies (Santin et. al., 1999). However, it is the hippocampal formation that has received the most attention. Thus, the discussion here will focus on this structure alone.

The Hippocampal Formation

Figure 1

Rat Hippocampus



The hippocampal formation is a collection of several structures, both cortical and subcortical. Cortically, the entorhinal cortex (EC) is the primary input to the subcortical structures, and receives afferents from the perirhinal cortex, the retrosplenial cortex and the medial frontal cortex (Amaral & Witter, 1995). Furthermore, the hippocampal

formation as a whole is also subject to projections from subcortical areas such as the hypothalamus, the locus coeruleus, the raphe nucleus and the ventral tegmental area, and providing information to the thalamus, hypothalamus and the olfactory regions. Subcortically, the hippocampal formation consists of the hippocampus proper (HPC), which is further broken down into several fields, the dentate gyrus (DG), and the subicular complex. The hippocampal formation as a whole has been extensively studied, and much is now known about the intra-hippocampal circuitry. While the EC sends its efferents to all areas of the HPC, the most studied of them is the so-called perforant pathway, which is a collection of fibres travelling through and terminating within the dentate gyrus (Amaral & Witter, 1995). The DG also receives input from the medial septal nuclei (Jakab & Leranth, 1995) as well as the brainstem and the supramammillary region (Amaral & Witter, 1995). The only output from the DG, however, comes from the granule cells of the granule cell layer of the DG (GCL-DG). These projecting mossy fibres terminate within the CA3 field of the HPC. The only known extra-hippocampal projection from the CA3 is to the septum, both medial and lateral nuclei. Within the HPC, the CA3 pyramidal neurons send their Schaffer Collaterals to the CA1. This field of the HPC projects internally to the subicular complex, as well as to several cortical regions, primarily the EC. The subiculum, whose divisions will not be further discussed, sends its outputs to the para- and pre-subiculum, as well as to the EC and other cortical areas.

Each of these primary structures has been studied in kind, and a fair amount of data has been compiled in regards to the relative contributions of these individual areas to spatial/navigational abilities in the rat. Lesions of these areas have been the primary means of determining their functional aspects, and that data will be briefly examined below.

Lesion Studies

The CA3 Field

Some of the early work detailing the cognitive deficits arising from damage to the CA3 has come from Ian Whishaw and Brian Kolb. Specifically, it was determined that bilateral, but not unilateral kainate induced lesions of CA3 resulted in an increased escape latency from the MWM, though the animals did eventually learn to perform the task, albeit suboptimally (Sutherland et al., 1983). Again, Whishaw (1987) showed that kainite-induced lesions of the CA3-CA4 region of the hippocampal formation decreased performance on the MWM relative to controls. However, these animals were not as impaired as those that had received lesions of the DG, suggesting that the DG may be more important for spatial abilities. One problem with these papers, however, is that both of them lesioned not only the CA3, but what those authors called the CA4. It has been suggested that the CA4 is not a part of the hippocampus proper, but is actually the hilus, or polymorphic layer of the DG (Amaral & Witter, 1995). It is entirely possible that the deficits seen in these rats, then, were due to a compromized DG as opposed to damage to the CA3, *per se*. However, Stubley –Weatherly and colleagues (1996) noted that kainite-induced lesions localized to the CA3 field, and destroying approximately 88% of the pyramidal cells there, resulted in specific impairments in the ability of the animals to learn the task. More recently, the Mosers have examined the role of the hippocampus in spatial behaviours. In one study, the CA3 of the dorsal HPC was transected through the transverse plane (Steffenach, Sloviter, Moser & Moser, 2002). Rats with these lesions showed impaired retention of pre-surgical MWM training, as well as increased trials required to learn a new MWM task.

Dentate Gyrus

The dentate gyrus has received somewhat more attention than the CA3, due to the discovery of preferential damage to the granule cells of the DG following intraventricular microinjections of the axoplasmic transport inhibitor colchicine, a known neurotoxin (Goldschmidt & Stewart, 1980). Sutherland et al. (1983) compared DG-

lesioned rats to sham-operated controls in the MWM and found that the lesioned animals did not exhibit any learning whatsoever. Moreover, they also discovered that DG-lesions caused greater impairment in this spatial reference task than did damage to the CA3 field, as noted above. Xavier et al. (1999) further examined the relative role of the DG on a water maze. They discovered that rats with bilateral DG lesions performed significantly worse than sham-operated controls on measures of path length and escape latency. This was tempered, however, by the fact that their lesions included both the GCL-DG, the hilar region of the DG and destroyed approximately 23% of the CA1 pyramidal neurons. Thus, deficits in this region may have been partially attributed to this latter field, as opposed to the DG *per se*.

The CA1 Field

O'Keefe and Dostrovsky (1971) postulated that the hippocampus functions as a spatial mapping system, noting that certain directional orientations caused a spiking in individual CA1 pyramidal cells that did not occur in those cells in other directional orientations. This led other investigators to examine the effects of CA1 lesions on spatial reference memory. Stubley-Weatherly et al. (1996) found that kainite-induced lesions of the CA1, in which approximately 50% of the pyramidal cells were destroyed, produced profound deficits on the ability of those animals to learn the submerged-platform water maze. Convergent evidence comes from Gilbert, et al., (1996), who reported that the acquisition of the MWM was also impaired, relative to controls, following kindling to the CA1 field.

Subicular Complex And The Entorhinal Cortex

The subiculum (referring to the subiculum proper, pre- and para-subiculum) has received less attention than the other areas of the hippocampus. Given the role of this area in hippocampal processing, however, this seems unfortunate. The subiculum is the final subcortical information processing area of the hippocampal formation, and is directly connected to the CA1, the EC and the other cortical structures which provide input to the hippocampal formation (Amaral & Witter, 1995). One paper examining the effects of subicular lesions on the reference memory of rats using a MWM comes from

Taube, et al., (1992), who found that acquisition of this task was impaired. More detailed investigations into the functional role of this hippocampal complex will hopefully be forthcoming.

As with the subiculum, there has been a dearth of literature surrounding the effects of EC lesions on spatial reference memory. Bannerman and colleagues, however, have examined this phenomenon and concluded that the EC does not play a role in the processing of spatial behaviour, as measured using the hidden platform MWM task (2001). This contrasts with the findings of Roof et al. (1993), who noted that male rats did show a deficit in this same task. Intriguingly, Roof and colleagues used only unilateral EC lesions, whereas the lesions in the Bannerman study were bilateral, and thus should show greater impairment, if one indeed exists. It is tempting to think that some difference in the protocols or in the particular rat strain used in these studies may explain the contradictory results. As such, however, there is little other data to hint at the role that this area may play in spatial information processing, if any at all. Convergent evidence for the idea that the EC may not be involved comes from a study examining the effects of bilateral lesions on reference memory using the RAM. Galani and his colleagues (2002) found that EC-lesioned animals did not differ from sham-operated controls on either reference or working memory.

Gonadal Hormones and the Hippocampus

Hormonal Control Of Sexual Dimorphisms In The Hippocampus

Given that the hippocampus is known to mediate spatial learning and memory and that there is an apparent sex difference in this behaviour, investigations into putative dimorphisms in the structure of the hippocampus across genders has also been explored. Roof and Havens (1992) noted that the thickness and width of the granule cell layer of the dentate gyrus (GCL-DG) were larger in males than in females, specifically noting a laterality effect, in which this difference was most pronounced when comparing the right GCL-DG from males to the GCL-DG of either hemisphere in females. Further, the authors claim that this difference was still present when brain size was used as co-

variate, though they did not show those data. Evidence from meadow voles also suggests that there may be a sex difference in the right GCL-DG (Galea et al, 1999). This study corrected for the overall brain weights and hippocampal volumes when making comparisons of this layer. It was found that the right GCL-DG did not differ between the two groups when these factors were accounted for. However, when hormone titers were considered, differences did emerge; specifically, males with high androgen levels had a significantly longer GCL than did females with either high or low estradiol. This agrees well with the Roof and Havens' (1992) finding that the GCL width was testosterone sensitive in Sprague-Dawley rats. In contrast, Isgor and Sengelaub (1998) were unable to detect a sex difference in the GCL-DG, however, though they were measuring the overall volume of this layer. However, reports have indicated that there are more granule cells in the male dentate than in the female (Madeira, et al., 1988), so some sex differences clearly exist within this structure.

The CA3 and CA1 pyramidal cell layers (PCL) have also been investigated with regards to sexual dimorphisms. Specifically, Isgor and Sengelaub (1998) noted that the PCL volumes of both fields were larger in males than in females. They also noted a larger soma size in the pyramidal cells of these two fields. A different pattern of hormonal control was observed, though; in the CA1, ER activity could explain the morphological differences, while this was not the case in CA3. In the latter field, EB had no effect on soma size or PCL volume, but the non-aromatisable androgen DHT did cause the masculinization of these measures in females. Certainly, AR activity has been found to increase soma size in neural populations that are not sensitive to estrogens (Watson, et al, 2001). Isgor and Sengelaub have also noted that CA3 pyramidal cells have a greater volume of influence and longer dendrites in the high testosterone groups, which again, agrees well with previous work by Madiera, et. al., (1991), showing that the volume of the mossy fibre system was larger in males than in females. Unfortunately, Isgor and Sengelaub did not compare the relative size of these structures, as controlled for by Galea et al., (1999)

Localisaton Of AR & ER In The Hippocampus

To help understand the physiological effects of gonadal hormones, several studies have attempted to localize AR and ER within the hippocampus, both perinatally and in adulthood. A comparison of different studies using mRNA *in situ* hybridization, tritiated hormone binding assays and immunolocalization of receptor proteins reveals the presence of substantial AR & ER levels within the CA1 and CA3 fields, and to a lesser degree, the dentate gyrus.

ER Localisaton

Some early reports examining radiolabeled estradiol concentrations in the hippocampus were varied, with Pfaff (1968) showing relatively high levels of the radiolabeled estrogen, ^{3}H-E2, in the hippocampus, while Stumpf and Sar (1976) were unable to replicate that result. In 1988, Loy and colleagues re-examined the uptake of ^{3}H-E2, finding that while there was a relatively low uptake, it was localized to specific areas of the structure, specifically to the ventral regions of the CA1 and CA3, and with what appeared to be interneurons preferentially labelled. In 1990, Simerly et al. used an *in situ* hybridization technique to examine ER mRNA in the adult brain. Again, a very modest signal was obtained from the various areas of Ammon's Horn, primarily within the PCL. Using ER-specific antibodies, Weiland et al., (1997) also replicated the finding of light ER distribution within the hippocampus, also noting that they were principally found in the interneurons. They also showed that ER-immunoreactivity (ir) was denser in the CA1 than the CA3. Recent studies have found that ER-ir is not localized solely to the interneurons but may be found in the astrocytes or, to a lesser extent, the pyramidal cells within Ammon's Horn (Milner et al., 2001; Solum & Handa, 2001; Hart et al., 2001), not just in adults (Milner et al., 2001; Hart et al., 2001), but also in neonates (Solum & Handa, 2001). It has also been suggested that pyramidal cell ER-ir is primarily in the ventral regions, with interneuron labelling found throughout the entire extent of the hippocampus (Hart et al., 2001). These studies agree well with a previous report that a new radiolabeled estrogen, [^{125}I]-estradiol, is found in the pyramidal cells of both CA1 and CA3, as are the mRNA's of both isoforms of the estrogen receptor (Shugrue &

Merchenthaler, 2000). This study also determined that pyramidal cell labelling was greater in the ventral sections.

AR Localisaton

AR localization has been less well studied. Stumpf & Sar (1976) initially found very little tritiated-androgen ([^{3}H]-T or -DHT) uptake in the adult hippocampus, but a pair of studies in 1990 suggested that AR could be found in the adult telencephalon. Sar et. al. (1990) used a pair of antibodies to examine AR-ir, and found staining within the CA1, while Simerly et al. (1990) found AR mRNA in both the CA1 and CA3 regions, as well as in the dentate gyrus. McAbee and DonCarlos examined the neonatal hippocampus using *in situ* hybridization and discovered the presence of AR mRNA in the CA3 and CA1, though CA1 staining appeared to be greater (1998). The antibody PG-21 was then used to detect AR-ir in the adult (Xiao & Jordan, 2002). Again, the CA1 appeared to have greater staining than CA3, though in both fields, staining was prominent in the pyramidal cells. AR-ir or mRNA was not found in the DG in any of these studies. Taken together, these studies suggest that the CA1 and CA3 fields are responsive to both AR and ER, though the DG is likely responsive to ER, but not AR.

Physiological Effects of Gonadal Hormones in the Hippocampus

Estrogen

17β-estradiol has been well studied with regards to its developmental role, as a result of the strength of the aromatization hypothesis. E2 has been found to affect the proliferation of embryonic neural stem cells (NSC) *in vitro*, either enhancing cell number (E2 alone) or decreasing it (when paired with extracellular growth factor) (Brannvall et al., 2002). Furthermore, it was noted that the differentiation of the embryonic NSC was affected, with E2 promoting a neuronal, as opposed to glial, phenotype (Brannvall et al., 2002). These authors were able to determine that this effect is likely due to the ERα, as opposed to ERβ, due to the lack of immunostaining for the latter receptor subtype (2002). Interestingly, it was noted that ER subtype may in fact determine whether E2

has a pro- or anti-apoptotic effect in developing neural cells. Nilsen et al., (2000) found that hypothalamic cell lines in estradiol-containing cultures that expressed both ERα and ERβ saw increased survival rates, but those cell lines which expressed only ERβ were subject to greater cell death. Those cells that did survive had a greater expression of the anti-apoptotic mitochondrial protein, Bcl-2 and did not express FasL, the pro-apoptotic receptor for the Tumor Necrosis Factor-family member Fas (Nilsen et al, 2000). Overall, the interaction of ER subtype and other factors within the cell likely determines the fate of that given neuron. Other important genetic effects of E2 may also play a role as well, such as the increase in the mRNA of brain derived neurotrophic factor (BDNF) in castrated neonatal male rats given E2, as compared to those given vehicle alone (Solum & Handa, 2001), and the decrease, *in vitro*, of Nip2, a pro-apoptotic protein, following E2 application (Brusadelli et al., 2000).

Aside from cell fate, E2 is also known to mediate neurite outgrowth. Much of the work in this regard has been done using cultured hypothalamic neurons taken from late gestational rat fetuses. This paradigm has typically found that primary neurite numbers are relatively insensitive to E2 application, though there is a sex difference in their development (Carrer & Cambiasso, 2002). Once again, though, there is an interaction of E2 effect on this parameter when other factors are considered. For example, male hypothalamic neurons *in vitro* only see increased axogenic growth in response to E2 application when the cultures are also applied with a target-specific (heterotopic) glial preparation, as opposed to a homotopic glial preparation or no glial preparation (Cambiasso et. al., 1995). Furthermore, tyrosine receptor kinase B (trkB) receptors were expressed more in those neurons exposed to heterotopic glia, than those exposed to homotopic glia (Carrer et al., 2003). This suggests that there are target-specific glial growth factors secreted during development that interact with E2 and other neurotrophic factor systems to promote the axogenic effect of estradiol.

Dendrite branching has also been found in response to E2 application. Hippocampal neurons taken from rats at E18 show increased dendritic branching in response to E2 (Audesirk et al., 2003), and it is worthwhile to note that in this preparation, only ERα were present, with no ERβ staining apparent.

Aside from these developmental activities, E2 has well known effects in the adult brain, much of the research here utilising the female estrus cycle. In 1990,Gould, Woolley, Frankfurt and McEwen showed that ovariectomy (OVX) of adult females resulted in a significant decrease in the volume of dendritic spines of the CA1, but not CA3, pyramidal cells, and that this loss was prevented by the administration of E2 and P. Following this, Woolley et al., (1990) further discovered that the estrus cycle of female rats also sees the fluctuation of these spines in the CA1, but not CA3, with an approximate 30% fewer spines on the day of estrus (when E2 and P are low) than on the day of pro-estrus (when E2 and P are high). Further work showed that P activity can modulate these rapid changes, providing a larger peak amount of spines following E2 application, but also a much quicker reduction in spine density than would normally be seen (Woolley & McEwen, 1993). Since then, much of the work has examined the mechanisms by which E2 may provide these effects. Briefly, it has been found that E2 may act by increasing N-methyl-D-aspartate (NMDA) receptor (NMDAR) mRNA and protein (Gazzaley et al., 1996), NMDAR-dependent Ca2+ flux within CA1 neurons (Murphy & Segal, 1996), NMDA-dependent excitatory post-synaptic potential's (EPSP's) (Woolley et al., 1997; Foy et al., 1999), as well as the disinhibition of CA1 pyramidal cells concomitant with a decrease in glutamic-acid decarboxylase (GAD)-65 immunoreactivity in CA1 afferents (Rudick & Woolley, 2001), suggesting that estradiol can down-regulate the amount of GABA available for release in these cells. Estrogens have also been found to alter the phosphorylation of both the NR2 subunits of the NMDAR, and the extracellular regulated kinase-2(ERK2) (Bi et al., 2001), and can induce a phasic pattern of Fos expression within the CA1, CA3 and (to a much lesser extent) dentate gyrus (Rudick & Woolley, 2000).

Androgen

Many of the sites that have been examined with regard to the developmental effects of testosterone in the CNS have focused on areas involved in reproductive behaviours (such as the pre-optic area) which are mediated by estrogens (Dohler et al., 1984), while sites examined for the role of the AR have typically been peripheral, such as the external genitalia or the Wolffian reproductive tract. Some work on the AR in the

CNS has been done, however, androgen receptors have been found to prevent early developmental cell death in a number of different neural populations, including the visual cortex (Nunez et al., 2000) and the spinal nucleus of the bulbocavernosus (SNB) (Nordeen et al., 1985). The SNB has been a site of particular interest, given that the motor neurons of this nucleus innervate penile muscles (and are vestigial in females) (Breedlove & Arnold, 1980), show intense AR-ir (Freeman et al., 1995), and do not accumulate estrogens (Breedlove & Arnold, 1980). And while much of the work has focused on the development of this structure, little is known of the mechanisms by which the AR works to prevent cell death, except that it must act within the target musculature and likely involves the up-regulation of various retrograde trophic factors which act to prevent cell death during the development of the system. Most of the work in this structure has focused on the adult rat, and has examined the regulation of gene transcription within both the motoneurons themselves, as well as the target structures. It has been found that AR can regulate the expression of proteins such as β-tubulin and β-actin (Matsumoto, 1994), N-cadherin (Monks & Watson 2001), calcitonin gene-related peptide (CGRP) (Monks et al., 1999), ciliary-derived neurotrophic factor receptor-α (Forger et al., 1998), and is also responsible for maintaining the size of the SNB motoneurons (Watson et al., 2001).

In the hippocampus, androgens have been found to increase the excitability of CA1 pyramidal cell neurons (Smith et al., 2002), and like estrogens, androgens acting via the AR can increase the spine density of these cells in both males (Leranth et al, 2003) and females (Leranth et al., 2004).

SEXUAL DIFFERENTIATION

Peripheral Differentiation

Prior to the sexual differentiation of the individual organism *in utero*, the embryo, regardless of chromosomal status, develops the primordial male (Wolffian) and female (Mullerian) reproductive tracts, bipotential gonads, and bipotential external genitalia. Genetic (XY) males contain on their Y chromosome a gene known as the sex determining region of the Y (SRY), whose gene product causes the differentiation of the medulla of the bipotential gonads into testes (for a review, see Tilmann & Capel, 2002). The functioning testes then begin to secrete two hormones; the C19 steroid testosterone (T) and the peptide Mullerian Regression Hormone (MRH). MRH causes the Mullerian reproductive tract to involute, while T acts to rescue the Wolffian system which would normally regress in the absence of this signal. Finally, T, upon its conversion to dihydrotestosterone (DHT) through the enzyme 5α-reductase, further acts to cause the bipotential genitalia to differentiate; again, the structures that develop into the penis and scrotum would become the clitoris and labia, respectively, without this androgenic signal (Wilson et al., 1981). All of the actions of these two androgens, T and DHT, occur by binding to the AR, the only known receptor for androgens, though indirect evidence has suggested the existence of a membrane bound receptor as well (DonCarlos et al., 2003). The AR is a nuclear receptor which causes the transcription of DNA upon being bound by its ligands and is a member of the steroid receptor superfamily (Chawla et al, 2001).

It has been said that because specific signals are required to result in normal male differentiation, whereas female differentiation occurs in the absence of these signals, female differentiation is the default pathway. However, there are still certain mechanisms required to fully differentiate organisms into a normal female phenotype. For example, the bipotential gonads must require specific signals for normal, female-typical differentiation of this structure to occur. Thus, the phenotypic female is not simply a default mechanism, but instead, comes about, like the male phenotype, from a specific

cascade of molecular events, many of which are still a mystery at this time (Cotinot et al., 2002).

Central Differentiation

The differentiation of the mammalian brain is a somewhat more complex process. It is known that there are specific differences between males and females in many of the neural populations within the CNS. It is also well known that different neural areas have their own critical periods of proliferation, migration and organization (Bayer & Altman, 1995), and so examining the differentiation of the brain along a male-female dichotomy is made even more difficult. However, one major difference that seems to occur between the CNS and the peripheral nervous system (PNS) is the mechanism by which testosterone acts to induce sexual differentiation. Whereas dimorphic structures in the PNS seem to be acted on by T or DHT working through the AR, the AR is not exclusively responsible for the differentiation of dimorphic structures within the CNS.

Many nuclei within the mammalian brain, including the hippocampus (Hojo et al., 2004) express the enzyme aromatase, which converts androgens into the C18 steroids, estrogens (Wagner & Morrell, 1997; Roselli et al., 1985). These estrogens then act via their own receptors, either ERα or ERβ, to produce the masculine phenotype for that particular nucleus. Circulating estrogens in the developing female do not masculinize those organisms due to the presence of α-fetoprotein, an estrogen binding protein that prevents those hormones from entering the CNS (Vannier & Reynaud, 1975).

Testicular Feminization Mutation

It does happen, however, that genetic mutations alter the differentiation of individual organisms. The testicular feminization mutation (*TFM*), resulting in androgen insensitivity syndrome (AIS), is a condition in which the gene coding for the AR is mutated, thereby producing non-functional AR. The AR gene is located on the X-chromosome, thus XX females carry this mutation.

Table 1

Punnett Square detailing the genotype of offspring of males and carriers

Male / Carrier	X	Y
X*tfm*	X*tfm*X	X*tfm*Y
X	XX	XY

Daughters of a carrier have a 50% chance of becoming a carrier, while XY males have a 50% chance of being affected by this mutation. As a normal XY male has only one X copy of the AR gene, the presence of this mutation results in androgen insensitivity. In this situation, the Y chromosome still allows for the determination of the testes, which work to produce both T and MRF. The MRF again results in the involution of the female reproductive tract, but as the AR are non-functional, T is unable to rescue the male reproductive system. Furthermore, the external genitalia are differentiated along female lines, as DHT has no functional receptor upon which to act. Phenotypically, then, X*tfm*Y males are completely female, indistinguishable from XX or X*tfm*X females. The testes are unable to descend from the abdomen, but become vascularized and produce normal levels of T during development (Chung & Allison, 1979) though in adulthood, *tfm*'s have higher levels of plasma T than normal littermates (Chung & Allison, 1979; Roselli et al., 1987) despite producing much less T in the leydig cells of the testes (Chung & Allison, 1979). Thus, normal levels of T are circulating throughout the organism and available for aromatization during development.

PROPOSAL

It is clear, then, that not only is there a sex difference in spatial learning, but that the hippocampus, one of the neural structures underlying this ability, is also sexually dimorphic. The aromatization hypothesis states that the masculinization of the hippocampus and the resulting differences in adult behaviour should be mediated by the effects of 17β-estradiol, the estrogenic metabolite of testosterone, acting via the ER. Some of the data would seem to support this model; however, recent challenges have emerged.

The *tfm*-affected male rat makes for an excellent opportunity to try and dissociate the relative effects of the AR versus the ER in the organization of the hippocampus and spatial learning. Because normal T levels are present in the *tfm* male, there should be normal amounts of E2 and subsequent ER activity, but little to no AR activity. The aromatization hypothesis, then, would suggest that the spatial performance of these *tfm* males should be male-typical, with a masculinized hippocampus as well.

The following study will examine this by comparing *tfm*-affected male rats to the wild-type males and females, and females carrying the *tfm* mutation in the MWM. The hippocampi of these animals will then be examined with respect to the absolute and relative size of the CA1, CA3 and DG, along with the primary cell layers of those areas.

METHODS

Animals

A total of 56 Sprague-Dawley (SD) rats between 70-80 days old, were used for the water maze testing; 22 female, 22 male and 12 *tfm*'s. Of the females, 8 were carriers of the *tfm* mutation, and 14 were WT, not carrying the mutation. All animals were group housed in a colony room at the Simon Fraser University (SFU) Animal Care Facility (ACF), with access to rat chow and tap water *ad libitum*. The room temperature was held constant at 21˚C, and a 12:12 light cycle was maintained, with lights on at 12:00 pm, noon. All behavioural testing was done in a separate room at the ACF, and was approved by the SFU University Animal Care Committee. Animals were treated in accordance with Canadian Council on Animal Care Guidelines.

Handling

All animals were handled for a total of five minutes per day, for the seven days preceding water maze testing. Animals were removed from their home cages, and placed on an elevated platform (as per Perrot-Sinal et al., 1996), similar to the one on which they would be placed during testing. On the last day of handling prior to testing, each animal was individually handled by the experimenter for the purpose of marking their tails with a non-toxic fabric marker, for individual identification. Furthermore, for each day during the trials, all animals were individually handled by the experimenter for tracking purposes. The tracking system used in this study is only able to detect dark colours against a light background, and as SD's are albinos, it was necessary to create dark markings on their heads and backs. To do this, a thick, non-toxic, xylene-free marker was used. Total handling time was approximately 3-4 minutes per animal per session.

Testing Room and Apparatus

The testing room was a rectangular area, measuring 467.36cm X 406.4cm. Due to the size of the room and other logistic concerns, the water maze was placed in the centre of this area. Specifically, there was 157.48 cm from pool edge to wall length wize on both sides of the room. By width, it was149.86 cm from pool to wall on one side, 104.14 cm on the other. There was an overhead camera used for tracking animals while in the pool. The camera was connected to a video monitor and a computer. Software (Chromotrack, San Diego Instruments, San Diego, CA) was used to track the animals' progress, and to calculate the time spent in each area of the pool. The pool itself was 151.13 cm in diameter. The water in the pool was made opaque by mixing in a non-toxic acrylic white paint. Water levels were 2.5 cm above the platform, and maintained at 23± 1°C. A white, opaque curtain surrounded the pool, blocking access to visuo-spatial cues and prominent landmarks within the testing room.

Water Maze Protocol

Each animal was tested four times a day for five days. The pool was broken up into 4 equal sections, arbitrarily considered to be north, east, west and south. The platform was randomly placed in the middle of the NE quadrant, and left in that location for the entirety of the testing period. The animals were released into the pool from each of the 4 starting locations every day in a pattern that was randomly determined prior to testing. For every trial, the animal was placed in the pool facing the wall. Animals were allowed 60 seconds to find the platform. If they were unable to find the platform in that time, they were guided to it by hand. They were allowed to remain upon the platform for 15 seconds, and were then removed. A minimum of 5 minutes elapsed between trials, during which time the animal was placed on an elevated platform in the testing room. A heat lamp was affixed above the platform. All testing was started at noon, and the order in which the animals were tested was randomly changed, to prevent any time of day effects.

Identification of Females and Tfm's

After all testing was completed, phenotypic female animals had to be genotyped. This was initially done by breeding the females. Approximately 21 days following birth, pups were removed, and searched internally. A *tfm*-affected male was determined by the following criteria: phenotypic female genitalia, small urogenital distance, internal testes, and no inguinal canal. Litters containing *tfm*-affected males indicated that the mother was a carrier of the mutation. In litters where there were no *tfm*'s, the mother was considered to be a wt female. Of the 34 phenotypic females, 18 were identified in this manner. The remaining 16 were identified through polymerase chain reaction for the AR gene product. This procedure was carried out at the Michigan State University (MSU), using tissue taken from the animals at the SFU ACF. For tissue removal, each animal was deeply anaesthetized using isoflurane, and then a tissue punch was used to remove two small patches of tissue from the ear. Tissue was sent to MSU by overnight delivery, packed in dry ice.

Histology

After identification (at approximately 6 months old), 15 animals (6 *tfm*'s, 5 males & 4 females, all age matched, littermate controlled) were killed by a CO2/O2 blend. They were then perfused transcardially using 0.1M PBS followed by 4% paraformaldehyde (PFA), with a ph of 7.4. Brains were rapidly removed and immersed in PFA for a further 2 hour postfix. Following this, the tissue was then transferred to a 20% sucrose solution (in PBS). Following cryoprotection, brains were sliced on a sliding microtome. All slices were 60 µm thick, and only every fourth section was used. The remaining tissue was suspended in DeOlmos solution and placed in a deep freeze. Tissue was mounted, and Nissl stained using thionin. Slides were cover-slipped immediately and left overnight to dry.

Water Maze Analyses

Repeated Measures F-tests were used for examining the distance to platform, escape latency, speed, and the percentage of time spent in the four quadrants and three

rings arbitrarily imposed upon the pool. Where overall F-tests were significant ($p \leq 05$), individual students t-tests were used to compare the groups for each trial. Where the two female groups, WT-females and carrier-females, did not differ, they were collapsed into one group, termed females. Students t-tests comparing both females and males and females and *tfm*'s were one-tailed. As there is already ample evidence for male superiority on spatial/navigational tasks, and *tfm*'s are hypothesized to have a male CNS due to aromatization, we believe that we are justified in this practice. Student's t-tests comparing males and *tfm*'s were two-tailed.

Morphological Analyses

Once the histology was complete, the volumes of the CA1, CA3 and the DG for each of the sexes was examined using the Analytical Imaging Station (Imaging Research Inc, St. Catharines, Ont.); this software allows for an estimation of the 3-dimensional volume of any structure, provided that there is a constant interslice interval within any tissue of interest (240µm in this analysis). The total hippocampal volume was estimated for each animal, as was the volume of the CA1, CA3 and DG, as well as the primary cell layers of each of these fields. The estimation of the hippocampus included the CA1, CA2, and CA3 fields, the dentate gyrus, the fornix, the nucleus of the dorsal hippocampal commisure (dhc), the dhc and the alveus, and the subiculum, including the parasubiculum and the postsubiculum. Cortical areas, including the entorhinal cortex, were excluded from the analysis. The final slice analysed for hippocampal volume estimation corresponded to approximately 7.04 mm posterior to the bregma, according to the atlas of Paxinos and Watson (1997). Figures 2 & 3 show the dorsal and ventral aspects of the hippocampus, respectively. For the assessment of dorsal regions, the last slice included corresponded to 4.52 mm posterior to the bregma, according to that atlas. A ratio of each area to the total hippocampal volume was then obtained. A one-way analysis of variance was then performed to establish any differences in these areas across sex. If the ANOVA was significant (at the .05 level), individual students t-tests were performed to determine which groups differed.

Figure 2:

Dorsal Hippocampus (Stereotaxic)

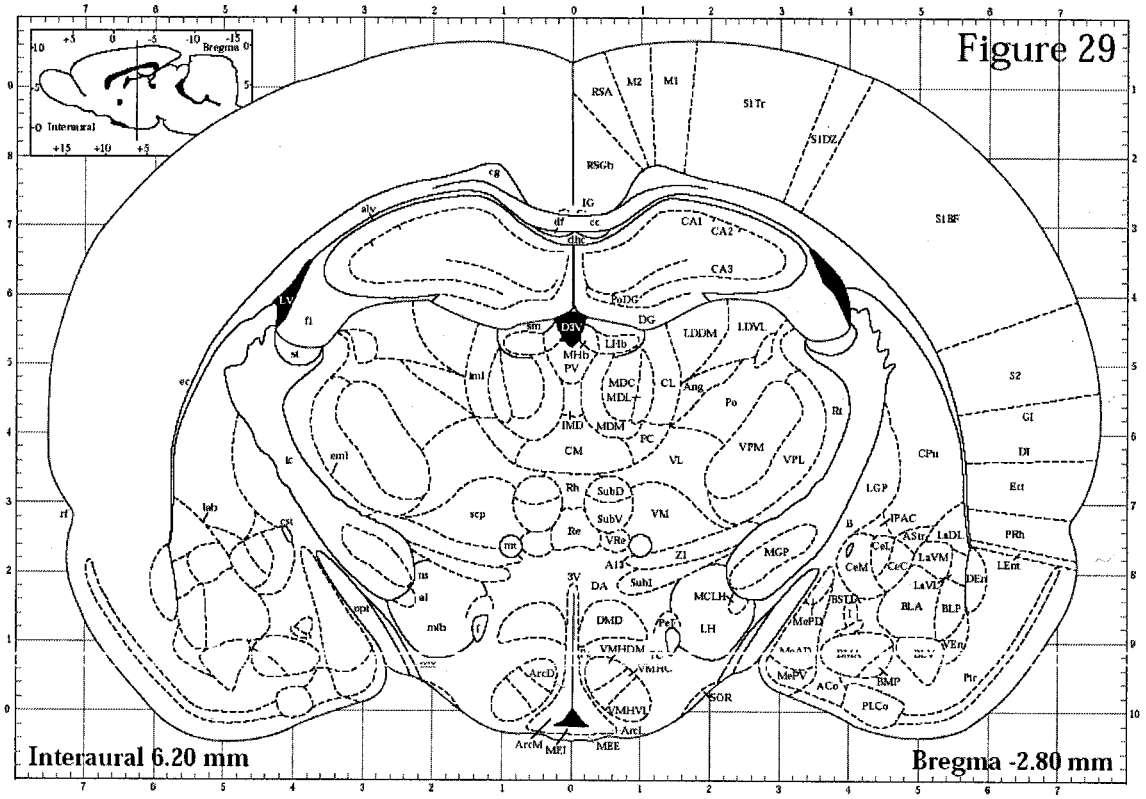


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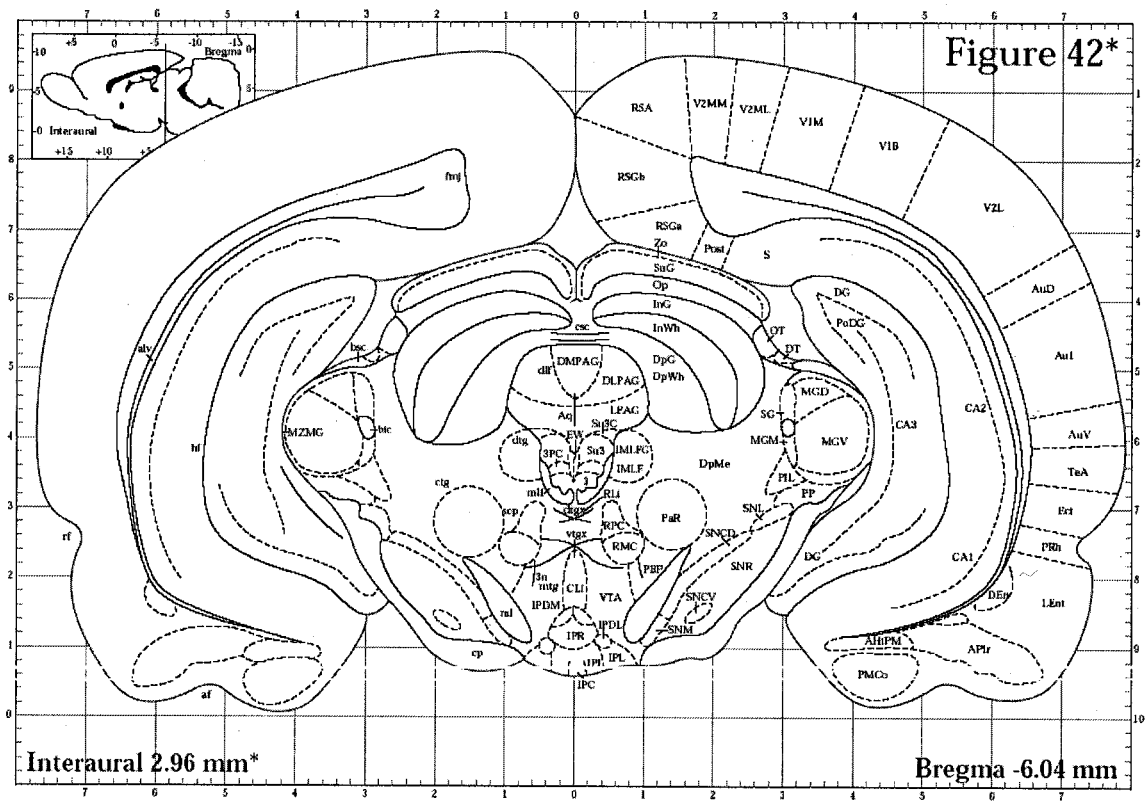
Figure 3:

Ventral Hippocampus (Stereotaxic)



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RESULTS

Morris Water Maze

Escape Latency

First, both groups of females were compared. The repeated measures F-test revealed that there was no difference between the WT and the carrier females (F=0.052, p=0.822). Thus, the two groups were collapsed together as females for all further analyses.

The overall repeated measures F-test examining escape latency for all three groups was significant (F=4.948, p=.011), an effect not due to speed (F=0.056, p= 0.945). Individual student's t-tests revealed that males and females performed similarly on days one and two, but the differences came in days 3, 4, and 5. On day three, males found the platform significantly faster overall (t=3.190, p=.002) (fig. 4) with trial-specific differences on trials 2 & 4 (t=1.681, p=0.05 & t=2.123, p=0.02, respectively) (Fig. 5). On day four, males were again faster overall (3.292, p=.001) with trial specific difference on trials 2 and 3 (t=3.247, p=0.001 & t=2.218, p=0.016, respectively) on that day (Fig. 6). Finally, on day 5, males were faster on the third trial (t=1.876, p=0.034) (Fig. 7).

Individual student's t-tests were also carried out to examine any potential differences between the *tfm*'s and either males or females. Males performed better than the *tfm*'s on day 3 overall (t=2.117, p=.036). On day four, *tfm*'s found the platform faster than females overall (t=2.139, p=.034), and specifically on the fourth trial (t=1.802, p=0.0405) on that day (Fig. 6). On day five, *tfm*'s again found the platform faster overall, (t=2.251, p=.026) and on the second trial (t=1.863, p=0.036), though by the last trial, all groups were performing equally well (Fig. 7).

Distance to Platform

The two groups of females, WT and carrier, were compared first, using a repeated measures F-test. The results show that there was no difference between these

two groups (F=0.104, p=0.751), and thus, they were collapsed together as female for all further analyses.

The overall repeated measures F-test examining the distance travelled to platform for all three groups was significant (F=3.302, p=0.045). Individual student's t-tests revealed that the difference between males and females occurred during the last three days. On day three, males were better on the fourth trial (t=1.954, p=0.0285), on day four, males performed better on trials two and three (t=3.056, p=0.002; & t=1.953, p=0.0295, respectively), and on day five, males travelled a shorter distance than females on the third trial (t=1.910, p=0.0315).

Individual student's t-tests were also carried out to compare the *tfm*'s with both males and females. Again, *tfm*'s did not differ from males at any time point, but did travel shorter distances on two occasions. On day four, *tfm*'s were better than females on trial four (t=1.898, p=0.0335), and on the second trial of day five (t=1.769, p=0.043).

Time spent in the Rings

As mentioned above, the pool was arbitrarily divided into three rings, to help analyse the pattern of behaviour while in the pool. These rings are termed the outer ring (OR), the middle ring (MR), and the inner ring (IR), which is the area directly in the centre of the pool. For all three areas, the WT and carriers were compared first. There were no significant differences in any of the comparisons (IR - F=0.344, p=0.564; MR - F=0.014, p=0.907; & OR - F=0.145, p=0.708), and thus, these groups were collapsed together as females. Overall repeated measures F-tests were performed to determine whether or not there were any differences in the percentage of time spent in any of these three different areas. These analyses determined that there were no differences across the groups. The results are, for the outer ring, F=1.668, p=0.198, for the middle ring, F=2.191, p=0.122, and for the inner ring, F=1.534, p=0.225.

Time spent in the Quadrants

Repeated measures F-tests were also performed to examine the percentage of time spent in the four arbitrarily assigned quadrants of the pool, called northeast (NE), northwest (NW), southeast (SE), and southwest (SW), and again, the WT and carriers

were compared first. As there were no significant differences in any of the comparisons (NE – F=1.365, p=0.256; NW - F=0.610, p=0.444; SE - F=0.001, p=0.981, & SW – F=2.401, p=0.137), these two groups were collapsed together as females. Overall, there were no differences for the percentage of time spent in the SE and SW quadrants (F=1.121, p=0.333; & F=1.531, p=0.226, respectively). The platform was located in the NE quadrant, and surprisingly, there was no difference in the overall F-test for this area (F=0.365, p=0.696). However, for the NW quadrant, there was a significant difference (F=5.503, p=0.007), with the females spending more time in this incorrect area than either the males or the *tfm*'s.

Figure 5:

Latency to Platform: Day Three

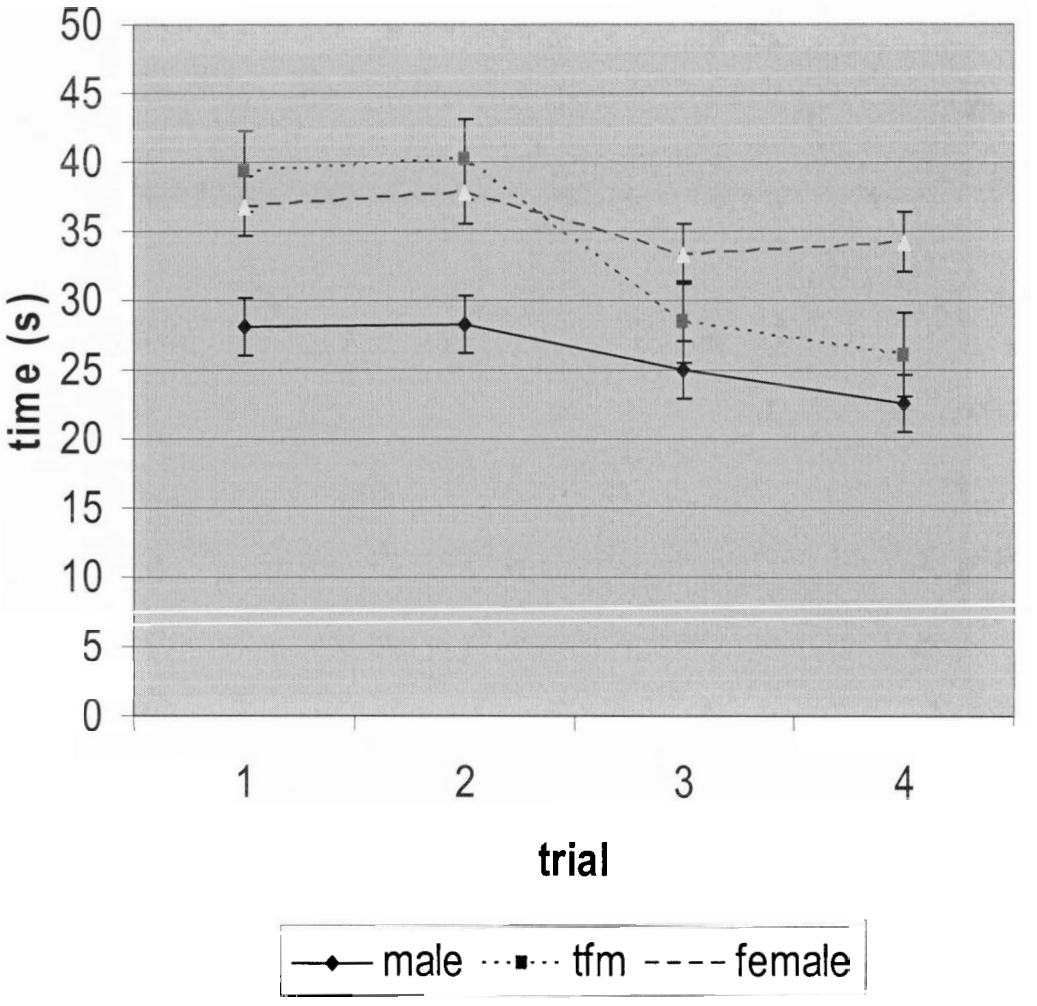


Figure 5:

Latency to Platform: Day Three

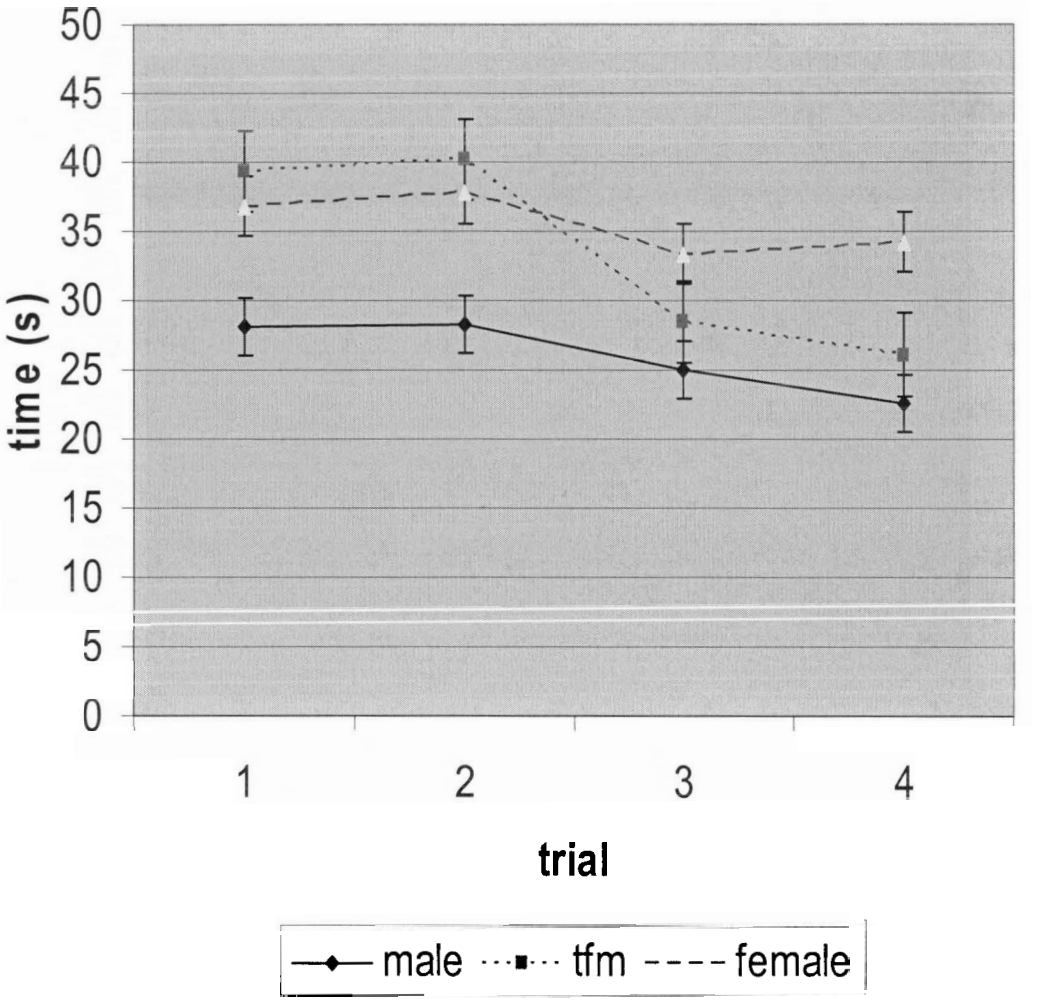


Figure 6:

Latency to Platform: Day Four

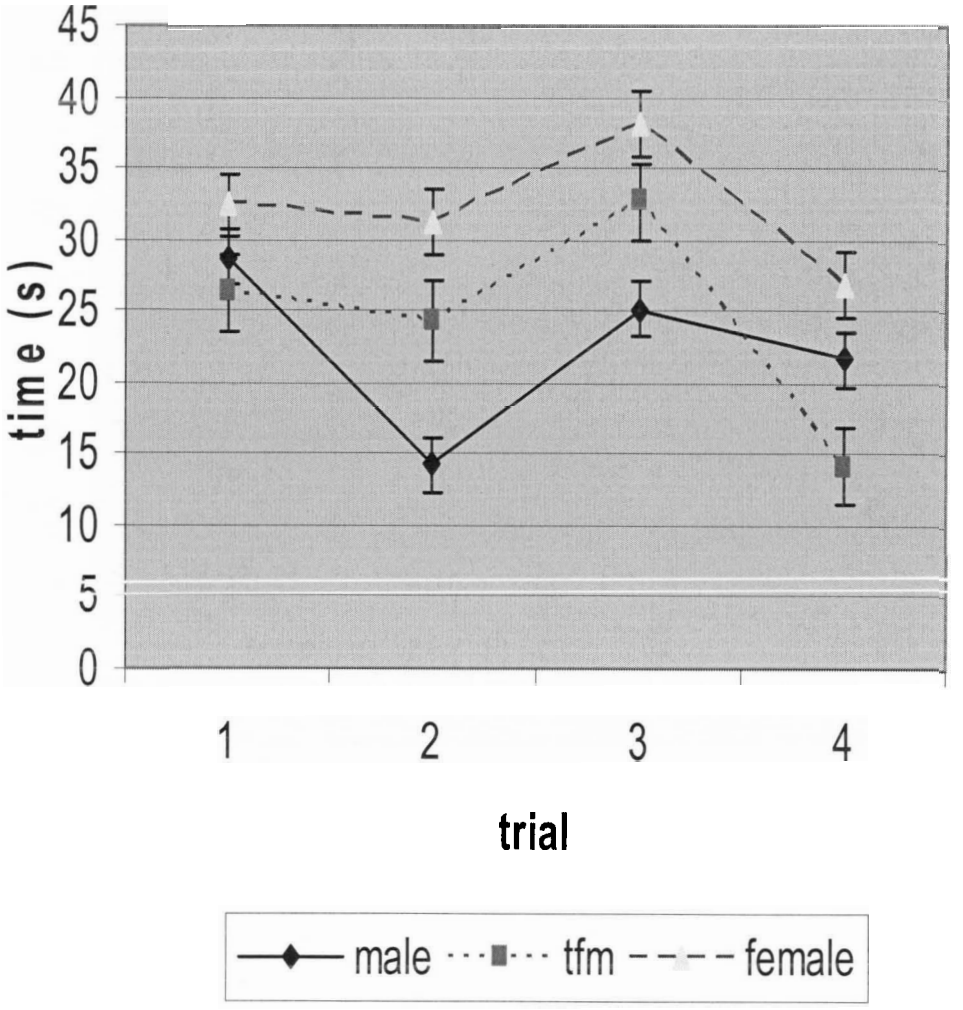
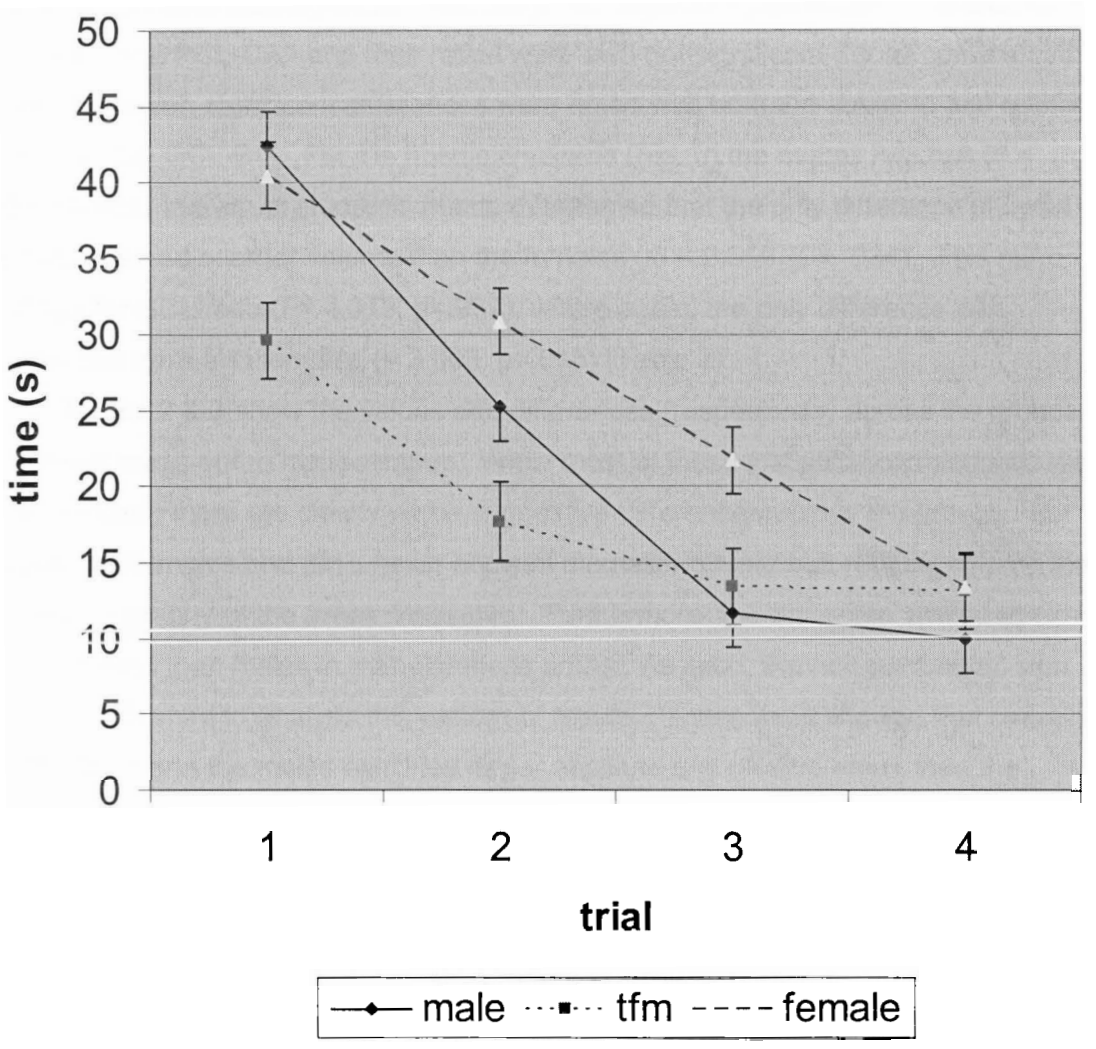


Figure 7:

Latency to Platform: Day Five



Hippocampal Morphology

Total hippocampal volume did not differ across the three groups ($p>.05$) (Table 2). Other comparisons included the CA1, CA3 & DG volumes, the dorsal areas of the

CA1 and CA3, and the primary cell layers of the CA1 & CA3 (PCL) and the DG (GCL). Finally, the volume of these areas was divided by HPC volume, and multiplied by 100, to determine relative size of these areas within the HPC. None of the CA3, dorsal CA3, or the PCL of the CA3 was significant, nor were the ratios for those areas. The DG, CA1, dorsal CA1 and PCL-CA1 and their ratios were also not significant (for all comparisons, $p>.05$). However, significant differences were noted with both the absolute and relative volumes of the GCL-DG. For the absolute volume (Fig. 6) the overall ANOVA ($F = 3.881$, $p=.05$). Individual student's t-tests determined that the only difference showed that the *tfm*'s had a larger volume than the females ($t8 = 2.559$, $p = .034$). This mirrored the effect for GCL ratio ($F= 4.373$, $p=.037$), where again, the only difference was between the *tfm*'s and females ($t=3.093$, $p=.015$) (Table 2).

Tables 2 & 3 show the values and differences (respectively) across the groups in the various areas of the hippocampus. While most of these statistical comparisons were non-significant, there are clearly some suggestive differences across the groups. For example, both males and *tfm*'s never showed a smaller absolute or relative volume than the females for any of the areas measured. Furthermore, the *tfm*'s also tended to exhibit larger volumes than males in many of these areas. As such, the non-parametric sign test was performed to analyse this pattern of results. These tests showed that overall, both the *tfm*'s and the males exhibited larger absolute and relative areas than the females (male > female, $p=.002$; *tfm* > female, $p=.002$), though the difference was not significant for males and *tfm*'s ($p=.077$). All sign test results were compared against $p=.0167$, to reflect the Bonferroni adjustment for post-hoc comparisons.

Figure 8:

Dorsal Hippocampus visualized with Thionin

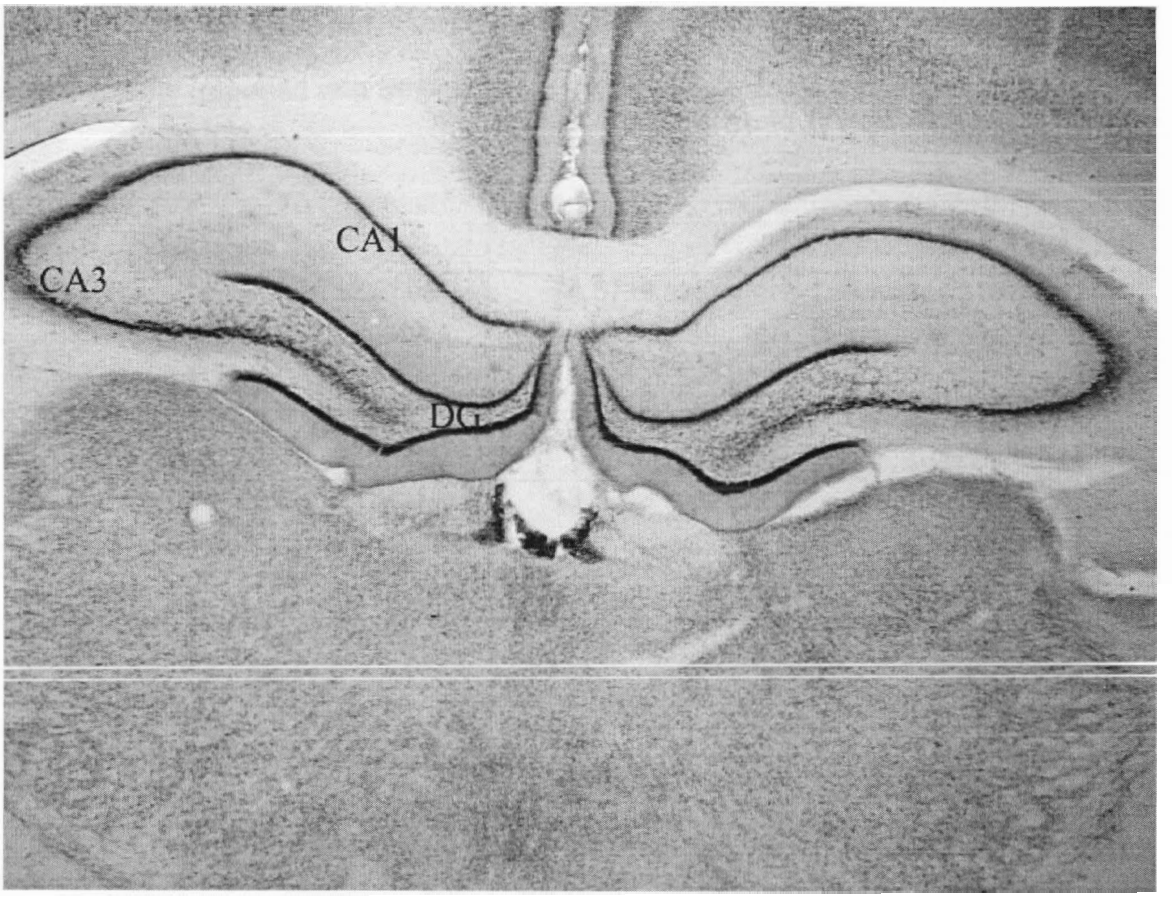


Table 2

Area Volume Measurements

Absolute volumes are in mm3, ratios are represented as a percent of the total hippocampal volume (reported with SEM's)

Area	Male	*Tfm*	Female
HPC	71.097±2.5808	74.57±0.8257	71.54±4.3181
DG	15.52±0.8066	17.56±0.8219	15.42±0.9587
DG%	21.84±0.86	23.57±1.06	21.54±0.12
CA1	12.86±0.7821	13.92±0.7082	12.37±0.9393
CA1%	18.1±0.9	18.69±1.02	17.26±0.5
DorsalCA1	8.41±0.2545	8.46±0.4007	7.37±0.865
DorsalCA1%	11.86±0.27	11.35±0.57	10.22±0.77
CA3	9.19±0.4384	9.46±0.2124	8.8±0.5668
CA3%	12.94±0.51	12.71±0.38	12.32±0.41
DorsalCA3	4.17±0.1877	4.26±0.1441	3.98±0.2618
DorsalCA3%	5.87±0.19	5.71±0.22	5.56±0.08
CA1-PCL	1.6±0.0545	1.68±0.0417	1.53±0.1312
CA1-PCL%	2.25±0.06	2.25±0.06	2.14±0.07
CA3-PCL	1.7±0.0528	1.87±0.0774	1.59±0.1857
CA3-PCL%	2.4±0.03	2.5±0.11	2.2±0.14
GCL	2.09±0.0989	2.35±0.1004	1.9±0.16
GCL%	2.95±0.13	3.16±0.12	2.64±0.08

Table 3

Differences in Hippocampal Volume

Area	XY (vs. *tfm*)	XY (vs. XX)	*tfm* (vs. XX)
HPC	-4.66%	-<1%	+4.24%
DG	- 13%	+ <1%	+13%
DG%	-7.4%	+1.4%	+9.4%
CA1	-7.6%	+4%	+12.5%
CA1%	-3.2%	+4.9%	+8.3%
DorsalCA1	-<1%	+14%	+14.8%
DorsalCA1%	+4.5%	+16%	+11%
CA3	-2.9%	+4%	+7.5%
CA3%	+1.8%	+5%	+3.2%
DorsalCA3	-2.1%	+4.8%	+7%
DorsalCA3%	+2.8%	+5.58%	+2.7%
CA1-PCL	-4.8%	+4.6%	+9.8%
CA1-PCL%	0%	+5.1%	+5.1%
CA3-PCL	-9%	+6.9%	+17.6%
CA3-PCL%	-4%	+9%	+13.6%
GCL	-11%	+10%	+23.7%
GCL%	-6.6%	+11.7%	+19.7%

Group comparisons are represented as the difference from the group in brackets

DISCUSSION

Morris Water Maze

The analyses clearly show a male superiority in the MWM, though the pattern of effects is somewhat intriguing. No difference was apparent within the first two days of testing, when one would expect to see a difference between the male and female groups, though a clear difference did emerge for the remainder of the testing period. In a similar protocol, a difference was discovered during the first two days of trials (Perrot-Sinal, Kostenuik, et. al., 1996), so the exact reasons for this discrepancy are unknown. Regardless, there is a clear sex difference between males and females in this paradigm, with females (both WT and carriers) performing poorly as compared to the males. The result from the *tfm* group is somewhat ambiguous, admittedly. A closer look at these results shows a particular pattern of performance. In the first two days, where there is no difference, all three groups were solving the maze equally well, reaching the 30 second levels for escape latency. The males were able to surpass this level of performance on the next day, approaching 20 seconds, whereas the females did not pass this level until the last day. The *tfm*-affected group also took longer to pass this plateau, but were able to do so on the fourth day, one day earlier than the females, and one day later than the males. *Tfm*'s thus showed an intermediate level of performance, behaving like females on the first three days, being significantly slower than males on day three and significantly faster than females on days 4 and 5, exhibiting trial-specific differences on the fourth and fifth days. This would suggest that the *tfm*'s are at least partially masculinized in their spatial behaviour. As there were no apparent differences in thigmotaxis, and the differences did not appear until the third day of testing, these differences appear to be due to genuine differences in spatial learning as opposed to some anxiety-related effect. The lack of complete masculinization in this behaviour in the *tfm*'s may result from the lack of AR activity, while the apparent superiority over the females may be due to the increased levels of estradiol available, as discussed above.

Hippocampus

The lack of a sex difference in the total hippocampal volume was somewhat unexpected, though not without precedent. Galea et. al., (1999) have found that hippocampal volume may differ only if hormonal status is taken into account in voles. Convergent evidence from another group using rats failed to find a sex difference in brain weight, despite the obvious differences in body size (Madeira et. al., 1991). Previous studies (Isgor & Sengelaub, 1998) have found that the PCL of both CA1 and CA3 showed a sex difference, with larger volumes occurring in males in both cases, which we were unable to replicate here (see table 2). Consistent with that study, we did not find a male-female sex difference in the GCL either, though we did note that *tfm*'s exhibited larger absolute and relative volumes within this layer than females, but not males. We also found that within other areas, specifically within the CA1 and DG, the *tfm*'s exhibited a higher volume than both males and females, though these differences were not significant. However, a sign test revealed that *tfm*'s consistently saw higher volumes, both absolute and relative, than females, as did males. This would suggest a male-typical pattern of hippocampal morphology. It is interesting to note that both the CA1 and DG exhibit the greatest amounts of ER-ir within the hippocampus of both males and females, while the CA3 tends to have lower levels of ER-ir (Solum & Handa, 2001; Hart et al., 2001). This suggests that the circulating levels of T may be available for aromatization within the hippocampus and subsequent estrogenic activity, however, no systematic analysis of ER-ir has yet been undertaken within the *tfm*-affected male. A recent study has shown, though, that immediately post-natal female hippocampi have significantly higher levels of estradiol than litter-matched males (Amateau et al., 2004). The exact significance of this finding with regard to hippocampal morphology needs to be elucidated, but clearly, the *tfm*-affected male needs to be analysed as well, so that a better understanding of the ER as well as AR on the development of this structure can be ascertained.

As mentioned, there were several differences across the sexes in some of the areas of the hippocampus that seemed fairly large, but failed to be significant (tables 2 & 3). It is possible that the small number of animals per group prevented significant findings here, which future work should address. However, a closer examination of the

results, particularly looking at those areas where the *tfm*'s were larger than males, shows that with the exception of the dorsal CA1, the males were always smaller than the *tfm*'s in the CA1 and DG measurements. This is interesting for two reasons. First, while the *tfm*'s and males have very similar measurements for the CA3, which is light in ER-ir, both the CA1 and DG are typically found to stain very densely for that antigen. Again, if T is generally more available for aromatization, owing to a lack of AR in the *tfm*'s, then this would suggest a strong role for E2 as trophic factor in the hippocampus. Secondly, the dorsal CA1 is thought to be more important than the ventral CA1 in spatial behaviour (Bannerman et al., 2003; Moser et al., 1995), and is typically lighter in ER-ir than ventral aspects of this area. As this is one area of the CA1 in which males are comparable to the *tfm*'s, it is interesting to speculate further about the role of E2 in the organization of both the hippocampus and spatial behaviour, especially given that the largest difference between males and females was also in the dorsal CA1.

GENERAL DISCUSSION

Though the results from the MWM showing an intermediate pattern of performance amongst the *tfm*'s could further challenge the aromatization hypothesis, one unknown is the regulation of aromatase within the hippocampus. As yet, no studies have examined the regulation of that enzyme in the hippocampus. However, while aromatase activity is androgen receptor dependent within the hypothalamus (Roselli et al., 1985), it has also been found that there is an androgen-independent regulation of this enzyme within areas of the limbic system, including the amygdala and the bed nucleus of the stria terminalus (Lauber et al., 1997). It is thus tempting to hypothesize that androgen-independent aromatase regulation occurs within the hippocampus as well. Indeed, it has previously been shown that within the medial and cortical amygdala, there is no difference in aromatase activity between *tfm*'s and control males (Roselli et al., 1987), even though within the medial portion of this structure, aromatase activity is slightly sensitive to androgen levels (Roselli et al., 1985). This would suggest that the available T within the hippocampus of *tfm*'s would be aromatized as per normal, and if typical ER activity occurs, then the difference in performance between males and *tfm*'s must be due to AR effects outside of aromatase regulation. Unfortunately, neither *in situ* hybridization nor immunocytochemical studies have been used to characterize the distribution of ER within these androgen-insensitive males. And while whole brain extracts from *tfm*'s suggest that there are identical levels of ER-ir in the *tfm* and male brain (Attardi et. al., 1976), discrete brain regions have not yet been examined, so it is unknown to what extent the ER is expressed within the hippocampus of *tfm*-affected males.

It is also important to remember that other limbic nuclei, as well as various cortical areas, are involved in spatial performance. So while the lack of morphological differences between the *tfm*'s and males seem to contrast with the apparent differences in water maze performance, there are a number of other regions that must be considered, including the septal nuclei, the amygdala, and the frontal cortex. Thus, a greater examination of these areas must determine the extent to which *tfm*'s may diverge from either males or females with regard to not only any potential morphological

differences, but also to dissimilarities along other lines, such as tyrosine hydroxylase- (Goldstein et al., 1992; Kritzer 1998, 2000) or choline acetyltransferase-immunoreactivity (Gibbs & Pfaff, 1992; Gibbs, 1996). These areas must also be examined with regards to their volume, as the posterodorsal medial amygdala has been found to differ across males, females and *tfm*'s, with the males being larger than the females, and the *tfm*'s showing an intermediate volume (Morris et al., 2003). Furthermore, as there is a well known interaction between the gonadal and adrenal steroids, whereby androgens down-regulate corticotropic releasing hormone secretions (Viau et al, 1999; Viau et al., 2001), and increased adrenal hormones are known to adversely affect learning (Lupien & McEwen, 1997), the cross-talk between these two endocrine axes should also be investigated within the *tfm*'s.

One limitation in this study is our inability to discern between organizational and activational effects, as all animals were gonadally intact during testing in adulthood. The prevailing belief, though, is that while the activational effects of gonadal hormones do influence cognitive abilities, circulating hormones in development are sufficient for organising this behaviour. Further work must clarify this distinction with respect to the androgen-insensitive male rat.

Another issue is that we did not examine any potential laterality effects. Previous work (Tabibnia et al., 1999; Roof, 1993b; Galea et al., 1999) has found that males show greater GCL-DG measurements in the right hemisphere and that the sexual dimorphisms in these animals is only apparent when comparing the right hemisphere of the males to the females right or left hemisphere, where the volume of the right hemisphere in the male is typically 8-9% larger than either in the female. An examination of Tables 1 & 2, however, will reveal that while there were non-significant differences in many of our comparisons, the size effects noted in our study where consistent, in many areas, with a difference of 8-9%, and even larger. While these were still considered non-significant using the ANOVA, the pattern and size of the results warrants further research into laterality effects and a possible interaction between that variable and sex.

In summary, we find that the *tfm*-affected male shows an intermediate pattern of performance on the water maze, seemingly female at the onset but reaching male-

typical performance earlier than females. The pattern of hippocampal morphology of the *tfm* animals appears to be male-typical overall. The water maze data challenges the aromatization hypothesis, suggesting that there may be an androgen-specific role in this behaviour. Given our results, and the recent findings of Amateau et al., (2004), the role of estradiol in the development of hippocampal morphology of normal animals, as well as the *tfm*, must be clarified in concert with a greater understanding of the function of the AR in the organization of this structure. Further research using other behavioural paradigms, such as the RAM, should be carried out to provide convergent evidence for the effects noted here, and to examine other potential sexually dimorphic abilities, such as working memory. Other dimorphic telencephalic structures should also be examined within the *tfm*-affected male.

LIST OF ABBREVIATIONS

°C	–	Degrees Centigrade
%	–	Percent
ACF	–	Animal Care Facility
AIS	–	Androgen Insensitivity Syndrome
ANOVA	–	Analysis of Variance
AR	–	Androgen Receptor
ATD	–	1,4,6-androstatriene-3,17-dione
BDNF	–	Brain Derived Neurotrophic Factor
CA1	–	Ammon's Horn 1
CA2	–	Ammon's Horn 2
CA3	–	Ammon's Horn 3
CA4	–	Ammon's Horn 4
CGRP	–	Calcitonin Gene-Related Peptide
CNS	–	Central Nervous System
cm	–	centimetres
CO2	–	Carbon Dioxide
DG	–	Dentate Gyrus
DHC	–	Dorsal Hippocampal Commisure
DHT	–	Dihydrotestosterone
DHTP	–	Dihydrotestosterone Proprionate
DNA	–	Deoxyribonucleic Acid
EB	–	Estrogen Benzoate

EC	–	Entorhinal Cortex
EPSP	–	Excitatory Post-Synaptic Potential
ER	–	Estrogen Receptor
ERK2	–	Extracellular Regulated Kinase 2
E2	–	17β-estradiol
E18	–	Embryonic Day 18
ERα	–	Estrogen Receptor-α
ERβ	–	Estrogen Receptor-β
Flu	–	flutamide
GAD	–	Glutamic Acid Decarboxylase
GCL	–	Granule Cell Layer
HPC	–	Hippocampus
ir	–	immunoreactivity
IR	–	Inner Ring
m	–	metres
mm	–	millimetres
MR	–	Middle Ring
MRH	–	Mullerian Regression Hormone
mRNA	–	messenger ribonucleic acid
MWM	–	Morris Water Maze
NE	–	North East
NMDA	–	N-methyl-d-aspartate
NMDAR	–	NMDA Receptor
NR2	–	NMDAR Subunit 2

NSC	–	Neural Stem Cells
NW	–	North West
OR	–	Outer Ring
OVX	–	ovariectomy
O2	–	Oxygen
p	–	probability
P	–	Progesterone
PBS	–	Phosphate Buffered Saline
PCL	–	Pyramidal Cell Layer
PFA	–	Paraformaldehyde
PNS	–	Peripheral Nervous System
RAM	–	Radial Arm Maze
s	–	seconds
SE	–	South East
SFU	–	Simon Fraser University
SNB	–	Spinal Nucleus of the Bulbocavernosus
SRY	–	Sex Determining Region of the Y Chromosome
SW	–	South West
trkB	–	tyrosine receptor kinase B
T	–	Testosterone
TFM	–	testicular feminization mutation
TP	–	Testosterone Proprionate
UM	–	University of Michigan
WT	–	Wild Type

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