SEX DIFFERENCES IN THE MOUSE VENTROMEDIAL NUCLEUS OF THE HYPOTHALAMUS

By

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ABSTRACT

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The following dissertation examined the ventromedial nucleus of the hypothalamus (VMH), a brain region that is sexually dimorphic in rodents. The experiments explored the role of circulating testosterone (T) and the androgen receptor (AR) in sex differences in the regional volume, neuron number, neuronal soma size, astrocyte number, and astrocyte complexity in the VMH of laboratory mice. The first experiment examined unaltered adult male and female mice to determine whether there are sex differences in the VMH. The results showed that the regional volume of the VMH is larger in males than females, and that this difference is accounted for by differences in the ventrolateral (VL) and dorsomedial (DM) subregions. The results also showed that males have more astrocytes than females in the VMH, and have more complex astrocytes than females in the VL subregion only. The next study examined the role of circulating adult T and AR in VMH structure in males, females, and males without a functional AR (induced testicular feminization or iTfm animals). The results showed that VMH volume, specifically of the DM and VL subregions, was greater in animals with circulating adult T regardless of sex or genotype, including iTfm males, suggesting that T acts on this measure through an AR-independent mechanism. Neuron number in the VMH was unaffected by adult T treatment, but was greater in males and iTfm males than females, suggesting that the sex difference in neuron number arises in development and is independent of both adult circulating T and AR. Adult T treatment

led to increased neuronal soma size in the VMH, and this response may be dependent on AR, as VMH somata were not significantly different between iTfms with or without T treatment. The last experiment examined whether there is a role of AR or circulating T in VMH astrocytes by examining males, females, and iTfm males with and without T treatment. The results show that males and iTfms did not significantly differ in the number of VMH astrocytes, and both had more astrocytes than females. In the VL subregion of the VMH, adult T treatment also resulted in more complex astrocytes regardless of genotype or AR status, while no differences in astrocyte complexity were seen in the DM subregion. Together, these experiments show that the VMH is indeed sexually differentiated in mice, with most of the differences observed in the VL and DM subregions. Additionally, many of these sex differences reflect sex differences in circulating T, and most of the sexual dimorphism in the VMH of mice comes about through an AR-independent mechanism.

To Quentin, and to all the others near and far who have helped along the way.

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CHAPTER 1: INTRODUCTION

INTRODUCTION

The following dissertation examines a brain region that is sexually dimorphic in laboratory rodents- the ventromedial nucleus of the hypothalamus (VMH). The following experiments examine the role of circulating androgens and the androgen receptor (AR) in regulating the regional volume, neuron number, neuronal soma size, astrocyte number, and astrocyte complexity in this brain region. In this introduction, I will discuss the background of sex differences in the rodent brain, rodent models used to study the role of the AR, the VMH, and astrocytes. I will conclude by discussing why it is important to study sex differences, and provide a preview of the following experiments.

Sex Differences and Hormones

In most vertebrate species, males and females differ in a number of ways, including many physical traits and behaviors. These sex differences include one of the most obvious- gonads. In males, the presence of the SRY gene on the Y chromosome will lead to development of testes, which will eventually begin to produce testosterone (T). In females, in the absence of SRY, the undifferentiated gonad will develop into ovaries. There are also other differences between males and females, ranging from behavioral differences, differential diagnoses of mental illnesses and, of interest to this dissertation, sex differences in the brain. Several areas of the brain are sexually differentiated, with the volume, neuron number, astrocyte number, and/or neuronal soma size differing between male and female rodents.

The traditional view of these sex differences in rodent brains is that high levels of T in males are aromatized to estrogen (E), and the brain is masculinized or defeminized via activation of estrogen receptors (ERs) during early development. This concept,

known as the aromatization hypothesis, posits that T is converted to 17-B estradiol (E2) which acts on estrogen receptors (ERs) to masculinize the brain (Naftolin, Ryan, Davies, Petro, & Kuhn, 1975). The aromatization hypothesis holds true for some sex differences in the brain and behavior of some species. One example of a sex difference in the brain that fits the aromatization hypothesis is the anteroventral periventricular nucleus (AvPV) of rats and mice, which is larger in females than males in adulthood. T promotes cell death in the AvPV through activation of ERs in male rats in early development (Arai, Sekine, & Murakami, 1996; Bleier, Byne, & Siggelkow, 1982; Sumida, Nishizuka, Kano, & Arai, 1993). In the SDN-POA of rats, T prevents perinatal cell death in the SDN-POA, which will therefore be larger in adult males than females (Gorski, 1986). In summary, some sex differences in the rodent brain follow the predictions of the aromatization hypothesis in that the early action of T through aromatization to E leads to masculinization or defeminization of the brain.

In addition to this traditional view of the aromatization hypothesis, there is evidence that the AR also plays a role in sex differences in brain and behavior, which can be studied using animals lacking AR. Such testicular feminization mutation (Tfm) animals are genetically male (XY) and have SRY, but carry an allele of *AR* that produces a dysfunctional AR protein and therefore can be used to address the role of the AR in sex differences when compared to wildtype (WT) males and females. Because the body cannot respond to the masculinizing influences of androgens, Tfm males have small, undescended testes in their abdomen and appear phenotypically female. Different spontaneous mutations in *AR* that lead to a nonfunctional AR were discovered in rats and mice. The Tfm mutation in rats has a single base pair replacement in the *AR* gene

that leads to a single amino-acid substitution resulting in a nonfunctional AR (Yarbrough et al., 1990). While a full protein is produced, the Tfm *AR* gene results in only 10-15% of the androgen binding as the wildtype *AR* gene. The naturally occurring spontaneous Tfm (sTfm) in mice also has a nonfunctional AR, but in this case it is dysfuncational as a result of a single base deletion that leads to a frameshift mutation (Charest et al., 1991). The sTfm mouse therefore produces a shortened AR transcript, resulting in almost no AR protein (Monks et al., 2007). The following studies will use yet another mouse Tfm model, the induced Tfm model (iTfm) that recapitulates the sTfm mouse using Cre-Lox technology, which will be described in further detail in later chapters.

When examining the role of AR using Tfm mice, it is also important to replace androgen levels as both sTfm and iTfm mice have low levels of circulating T in adulthood (Chen, Brummet, Lonstein, Jordan, & Breedlove, 2014; Zuloaga, Puts, Jordan, & Breedlove, 2008), which would also reduce the availability of T for aromatization to E. Indirect evidence suggests that T levels in sTfm mice are near the normal range in the *perinatal* period where T may have a large organizational effect on the brain (Goldstein & Wilson, 1972). Note that estrogen binding in the brains of Tfm mice and rats appears normal (Attardi, Geller, & Ohno, 1976; Olsen & Whalen, 1982), which is important when testing the effects of AR and circulating hormones in Tfm mice. However, aromatase activity may be decreased in the Tfm brain in rodents (Roselli, Salisbury, & Resko, 1987; Rosenfeld, Daley, Ohno, & YoungLai, 1977), which may prevent the conversion of T to E even if ER function is normal. In rats, providing T drives aromatase activity in the brain (Paden & Roselli, 1987), suggesting that replacing T will allow for aromatization to occur. However, if T increases aromatase activity by

activating AR, then equal levels of T in WT and Tfm males, of either species, may not equalize brain aromatase. Therefore, in experiments using Tfm rodents, it is important to consider equilibrating T levels to ensure that animals with AR and animals without AR are at least exposed to the same levels of T.

Some sex differences in the rodent brain seem to be partially mediated through the AR. The posterodorsal region of the medial amygdala (MePD) is a sexually differentiated brain region that is larger in males than females. Tfm rats have MePD volumes and soma sizes that are intermediate to WT males and females, suggesting that the AR is necessary for full masculinization of the region (Morris, Jordan, Dugger, & Breedlove, 2005), but that androgens still exert a partially masculinizing effect, presumably by producing metabolites that activate one or both ERs. Described in further detail below, the volume of the ventromedial nucleus of the hypothalamus (VMH) in Tfm rats is also intermediate to that of wildtype males and females, suggesting that the AR is required for full masculinization of the region. Several other brain regions show effects of the AR on sexually dimorphic brain areas, suggesting that the AR plays an important role in sexual differentiation in the rodent brain.

In addition to the role of AR, some sex differences in the rodent brain are dependent on circulating T in adulthood. Castration of adults may reduce the size of some sexually differentiated brain areas, while providing T through implants will lead to increased volume. One example of this type of sexual dimorphism is the MePD, which is larger in males than females. However, if males are castrated, this sex difference goes away (Cooke, Tabibnia, & Breedlove, 1999). If females are given T, the volume of the MePD increases to about male levels. Therefore, circulating T is important to replace in Tfm

mice in order to ensure that sexually dimorphic regions do not appear feminine simply due to lack of circulating T.

Taken together, this evidence suggests that both the AR and circulating hormones may play a role in sex differences in the rodent brain. Much more is known about Tfms and the role of AR in brain sexual dimorphism in rats than mice, but Tfm mice are of interest due to the availability of genetic tools and manipulations in this species. In the following dissertation, I will examine one sexually differentiated brain region, the VMH, in more detail in WT male, WT female, and iTfm mice in order to examine the role of the AR and circulating T in sex differences in this region.

The Ventromedial Nucleus of the Hypothalamus (VMH)

One sexually differentiated brain region is the VMH. The VMH consists of 4 subdivisions- the anterior (A), the dorsomedial (DM), ventrolateral (VL) and central (C) regions (Figure 1). The VL and DM subregions are associated with different behaviors, the majority of which are sexually differentiated (reviewed in (Dugger et al. 2007and Zuloaga et al. 2008). The VL is associated with sex behaviors in rats, such as lordosis and mounting behaviors (Kow & Pfaff, 1998; T. Matsumoto & Yamanouchi, 2000; Pfaff & Sakuma, 1979; Yahr & Greene, 1992). The VL has also recently been shown to mediate aggressive behavior in male mice (Lin et al., 2011). The DM is related to scent marking behavior and ultrasonic vocalizations in male rats (Harding & McGinnis, 2005). The DM and VL regions also have highest concentration of steroid receptors of all VMH subregions, in both rats (Simerly, Chang, Muramatsu, & Swanson, 1990) and in mice (our unpublished observations). Together, these qualities of the VMH make it an area of interest when studying sex differences and the role of the AR, as the region is involved

in several sexually differentiated behaviors and contains a high concentration of steroid receptors.

In addition to being involved with sexually differentiated behavior, the VMH is sexually dimorphic in volume. The VMH is larger in male rats than female rats (A. Matsumoto & Arai, 1983). Other research using Tfm rats suggests that the AR has a role in masculinization of the VMH, as the volume of the nucleus in Tfm rats was intermediate to males and females (Dugger et al., 2007). In this study, the VL subregion accounted for almost all sex difference in VMH morphology. Males had larger neuronal somata than did females, and Tfm males had smaller neuronal somata than WT males in the DM and VL. The VMH has not been characterized in this detail in mice, so I will examine the volume, neuron number, and neuronal soma size of the various VMH subregions in mice to see if there is sexual dimorphism, and whether AR plays a role in any dimorphism found.

Astrocytes

Another possible avenue for sex differences in the brain is in non-neuronal cells, including glia such as astrocytes. Astrocytes are characteristically star shaped and play many roles in the function of the brain. For example, astrocyte activity levels are closely related to blood flow in the brain, and this activity may in fact be the signal picked up in neuroimaging techniques such as MRIs (Figley & Stroman, 2011). Astrocytes also help with nervous system repair, metabolic support, structural support, and other functions. Increased astrocyte branch complexity has been associated with increases in functional synapses (Elmariah, Hughes, Oh, & Balice-Gordon, 2004; Pfrieger & Barres, 1997) and have also been associated with decreases in dendritic spines (Mong, Roberts, Kelly, &

McCarthy, 2001). Astrocyte branch processes may also be involved with the formation of new synapses and may be able to extend or retract to regulate contact between neurons (Hatton, 2002; Nishida & Okabe, 2007; Ullian, Sapperstein, Christopherson, & Barres, 2001). In summary, astrocytes play a large, and largely unappreciated, role in brain and synaptic function, and are another potential cell type that could play a role in any instance of neural plasticity, including sexual differentiation.

. Astocytes are also of interest when studying sex differences because they are hormone-responsive and have been shown to express steroid receptors. Some previous studies demonstrated hormone responsiveness of astrocytes (reviewed in Garcia-Segura & Melcangi, 2006) and also sex differences in astrocyte number and complexity in the rodent brain, including previous work from our lab in the rat medial amygdala (Johnson et al., 2008) and the mouse medial amygdala (Pfau, Hobbs, Breedlove, & Jordan, 2016) . Estrogens can also influence astrocyte morphology in the hypothalamus of fetal rats (L M Garcia-Segura, Torres-Aleman, & Naftolin, 1989) and in the hypothalamus of adult female rats (L.M. Garcia-Segura, Chowen, Dueñas, Torres-Aleman, & Naftolin, 1994)**.** Astrocytes have also been shown to express AR and ER in certain parts of the brain in several species, including rats (DonCarlos et al., 2006; Johnson, Schneider, DonCarlos, Breedlove, & Jordan, 2012) and guinea pigs (Langub & Watson, 1992). Other work in mice has also shown that neuroprotective effects of estrogens are mediated through ERs in astrocytes as selective ER knockout from astrocytes prevented these effects, while knockout of ER in neurons had no effect (Spence et al., 2011). These results suggest that steroid hormones may be able to act directly on astrocytes. In rats, estrogens influence the complexity of astrocyte arbors in

parts of the hypothalamus though indirect actions involving neurons (Mong, Nuñez, & McCarthy, 2002). This would mean that gonadal hormones may also act indirectly on astrocytes, by first acting on neurons that express AR or ER receptors. Together, astrocytes are hormone responsive, and steroid hormones may be able to influence astrocyte morphology directly or indirectly.

Although the VMH is well studied in regards to sexual differentiation, not much is known about astrocytes in the VMH, especially in mice. One previous study looked at VMH astrocytes in postnatal rats, and found that the astrocytes were relatively simplistic in structure and did not respond to testosterone propionate (Mong & McCarthy, 1999). However, as the VMH is known to be sexually dimorphic, it would be of interest to examine astrocytes in this region of the brain at later ages. The following experiments explore this question in the mouse VMH, and examine if there are sex differences in astrocyte numbers or morphology, and if the AR or circulating T influence these characteristics.

Importance of Studying Sex Differences

Studying sex differences in biological measures is an important part of the scientific process. Many past studies have chosen to study only males in pursuit of simplicity, claiming that estrous cycles in females complicate things and create additional variance (Beery & Zucker, 2011). However, Beery and Zucker (2001) also present several past studies that indicate that the idea that females, including mice, show more variance is not necessarily true and not a reason to exclude females from studies. As Larry Cahill writes, there is also a false assumption that for men and women

to be equal, they must be exactly the same (Cahill, 2014). However, treating males and females as exactly the same is not necessarily an effective strategy.

Many studies, including those testing drugs, have used only males, which doesn't take into account the potential for sex differences in effects, doses, or side effects of medication. NIH has made an ongoing effort to emphasize the importance of addressing sex differences, especially in preclinical research studies and in understanding sex differences in health and disease (Clayton & Collins, 2014; NIH, 2010). Therefore, studying sex differences at any level can help us understand these differences.

Summary

The following experiments examine the role of the AR and circulating T in sex differences in the VMH in the mouse brain. These experiments examine the VMH for sex differences in neuron number, neuronal soma size, astrocyte number, astrocyte complexity, and volume. I seek to establish whether there are sex differences in VMH volume in gonadally intact animals, examine the role of the AR and circulating hormones in any such sex difference, and explore potential sex differences, and the role of AR and T, in astrocyte number and complexity in the mouse VMH.

PREVIEW OF FOLLOWING CHAPTERS:

Chapter 2

In the first experiment, I look at unaltered WT male and female mice for sex differences in astrocyte number and complexity in the VMH. This experiment also looks for sex differences in VMH volume in unaltered male and female animals to confirm that VMH volume is larger in male mice than females. In the next experiment, I examine neuron number, neuronal soma size, and overall volume of the VMH in male, female, and iTfm mice that are all castrated and implanted with capsules that either contain testosterone (T) or nothing (blank). This experiment addresses both the role of circulating hormones and the AR in the dimorphic VMH. The last experiment looks at astrocyte number and complexity in male, female, and iTfm mice that are castrated and implanted with T or a blank capsule. Together, these experiments look at the role of AR and circulating T in regulating sex differences in the VMH.

Chapter 3

I provide an overall discussion of the previous experiments including their results and their implications.

CHAPTER 2: SEX DIMORPHISM AND STEROID SENSITIVITY OF THE MOUSE VMH

ABSTRACT

The ventromedial nucleus of the hypothalamus (VMH) is sexually dimorphic in rats, with males having a larger volume than females (Dugger et al., 2007). Rats lacking a functional androgen receptor (AR) have a VMH volume that is intermediate to that of males and females, suggesting that functional AR is necessary for full masculinization of the nucleus. To study sex differences and the role of AR in mice, we first looked at gonadally intact wildtype male and female mice sacrificed at postnatal (PN) day 90, and their brains stained for glial fibrillary acidic protein (GFAP) as a marker for astrocytes. We found that VMH regional volume is larger in males than females. As in rats, this sex difference in overall VMH volume in mice was accounted for by specific subregions, with the dorsomedial (DM) and ventrolateral (VL) subregions being larger in males. We also found more astrocytes in the VMH of male mice than in females. Males also had more complex astrocyte processes than females in the VL subregion only. Next, we examined the VMH in male, female, and induced testicular feminization (iTfm) mice lacking a functional AR. All animals were gonadectomized at postnatal age 60 (PN60) and implanted with either a testosterone (T)-filled or blank (B) capsule. Animals were sacrificed at PN90, and the brains sectioned and stained for stereological analysis. Overall volume of the VMH, and volume of the DM and VL subregions, was larger in animals treated with T, regardless of sex, and T treatment also increased VMH volume in iTfm mice. T treatment had no effect on the number of neurons in the VMH, but males had more neurons than did females. iTfm males were not significantly different

from wildtype males, suggesting that the sex difference in neuron number is independent of both adult circulating T and AR. T treatment also increased neuronal soma size in the VMH, which appears to be dependent on AR, as VMH somata were not significantly affected by T in iTfms. In a third experiment, a separate cohort of male, female, and iTfm animals were gonadectomized at PN60 and implanted with either a T or a B capsule, then perfused at PN90, specifically to examine effects on astrocytes. Males and iTfms did not significantly differ in numbers of VMH astrocytes, but both had significantly more astrocytes than did females. There was no effect of adult T treatment on astrocyte number, nor any interaction of genotype and hormone treatment on that measure. However, in the VL subregion only, adult T treatment resulted in more astrocytic process endings and branch points, regardless of sex or AR status, indicating that T's effects on VL astrocytes are independent of AR and hence may be mediated by aromatized metabolites of T acting upon estrogen receptors. Together, these experiments confirm that the mouse VMH is sexually dimorphic and androgen-responsive. Males have more astrocytes, with more complex processes, than do females in the VL subregion, where circulating T leads to more complex astrocytic processes. The volume of the VMH is largely independent of AR, as regional volume reflects circulating T levels even in iTfm males. The sex difference in neuron number in the mouse VMH is independent of AR or circulating levels of T. Neuronal soma size in the VMH was affected by circulating T, apparently mediated by AR. These results indicate that the AR plays a more limited role in the sexual dimorphism of the VMH in mice than in rats.

INTRODUCTION

Several regions of the rodent brain are sexually differentiated, including the ventromedial nucleus of the hypothalamus (VMH). The nucleus is located near the third ventricle in the hypothalamus and consists of 4 subdivisions- the anterior (A), the dorsomedial (DM), ventrolateral (VL) and central (C) regions. The DM and VL regions have the highest concentration of androgen receptors (ARs) within the VMH in both rats (Simerly et al., 1990) and in mice (our unpublished observations). The VL and DM subregions are also associated with several different behaviors, the majority of which are sexually differentiated (Dugger et al., 2007; Zuloaga et al., 2008). The VL is associated with sex behaviors, such as lordosis and mounting behaviors in females (Kow & Pfaff, 1998; Pfaff & Sakuma, 1979; Matsumoto & Yamanouchi, 2000; Yahr & Greene, 1992) and aggressive behavior in male mice (Lin et al., 2011), while the DM is related to scent marking behavior and ultrasonic vocalizations in male rats (Harding & McGinnis, 2005). These characteristics make the VMH a brain region of interest for studying sex differences and the role of hormones in behavior.

Previous studies showed that the VMH is sexually dimorphic in rats, and that the AR plays a role in this sex difference. The VMH is larger and has larger neuronal somata in male rats than females (Matsumoto & Arai, 1983; Dugger et al., 2007). Specifically, the VL subregion of the VMH accounts for the larger VMH volume in male rats compared to females, with the other subregional volumes not significantly differing between the sexes (Dugger et al., 2007). To examine a potential role of the AR in VMH sexual dimorphism, testicular feminization mutation (Tfm) rats, which are genetic XY males with a nonfunctional AR, were also examined. Tfm rats have an overall VMH

volume that is intermediate to that of wildtype males and females, and the VL subregion volume was significantly smaller in Tfm males than wildtype males and did not differ from that of females, suggesting that functional AR is necessary for full masculinization of the VMH in rats (Dugger et al., 2007). Tfm rats also had intermediate or feminine neuronal soma size in the VL and DM subregions. The fact that VMH morphology was not entirely feminine in Tfm rats suggests that at least part of the masculinization of this region is normally accomplished through an AR-independent mechanism, presumably via activation of estrogen receptors by aromatized metabolites of androgens.

As it is not known if the VMH is sexually dimorphic in mice, or if AR plays a similar role in sex differences in the VMH of this species, we examined these issues in the mouse VMH. First, we examined unaltered, gonadally intact adult males and females to see if the VMH is sexually dimorphic. Next, we examined the role of the AR and circulating androgens in the mouse VMH by analyzing the brains of male, female, and induced testicular feminization mutation mice (iTfm mice, described further below) that were gonadectomized and implanted with either a testosterone (T)-filled or a blank (B) capsule. Last, we examined the role of AR and circulating T on astrocyte number and complexity in the mouse VMH.

In the first experiment, we found there is indeed a sex difference in total VMH volume in gonadally intact mice, which is accounted for by sexual dimorphism of the VL and DM subregions. In this experiment, we also found a sex difference in astrocyte number and complexity, with males having more astrocytes than females, with more complex processes, but only in the VL subregion. We found that animals with circulating T have a larger VMH volume, even in iTfm animals lacking AR, suggesting this sex

difference in intact mice is due to the sex difference in circulating androgens, and that they affect the VMH independently of AR. Male mice also had more neurons in the VMH than females, and iTfm males did not significantly differ from wildtype males. Neuronal number was unaffected by T manipulations, suggesting that androgens act during development to permanently increase neuronal number in the VMH via a mechanism independent of AR. In contrast, adult T treatment led to increased neuronal soma size in the VMH in both sexes, an effect that may be dependent on AR, as VMH somata size did not differ between iTfms with or without adult T treatment. While adult T treatment had no effects on astrocyte number, it significantly increased astrocyte process complexity in the VL subregion of the VMH. Interestingly, T had this effect even in iTfm males, indicating that it is independent of AR.

METHODS

Experiment 1

Animal Procedures. Wildtype (WT) male and female mice were gathered from our ongoing induced testicular feminization mutation (iTfm) colony (described in more detail in the methods for Experiment 2). Females carrying one X chromosome with a recombined, and therefore dysfunctional AR, were crossed with WT C57/Bl6 males to create four genotypes; WT males (XY) and females (XX) were used for this study. All animals for all experiments were cared for in compliance with NIH guidelines for animal care and approved by the Michigan State University Institutional Animal Care and Use Committee. Animals were housed in plastic cages on a 12:12 LD cycle and had access to water and food pellets *ad libitum*. Animals were weaned at postnatal day 21 (PN21), ear punched for genotyping, and then group housed by sex.

PCR was used to genotype offspring by examining for Sry and the WT or recombined AR allele. Primers were targeted towards the sequence of DNA that included the lox sites and exon 2 of the AR gene. The primer sequence was AGC CTG TAT ACT CAG TTG GGG and AAT GCA TCA CAT TAA GTT GAT ACC and resulted in a band that was 400bp for the recombined AR allele, and 860bp for the WT AR. The two genotypes used for the current study were WT males (Sry positive and WT AR) and WT females (Sry negative and WT AR).

In this experiment, WT male and female animals were perfused at PN90 after an I.P. overdose of sodium pentobarbital (30ul/10g body weight). Once unresponsive, the chest cavity was opened and blood collected from the heart for T assays. Animals were then intracardially perfused with 0.9% saline followed by 4% buffered paraformaldehyde (0.1 M phosphate buffered saline (PBS; pH 7.4)). Brains were removed and post fixed in 4% paraformaldehyde for 2 hours, then transferred to 20% phosphate buffered sucrose. *GFAP Staining.* After the brains were infiltrated with sucrose overnight, they were sectioned on a freezing sliding microtome at 30um in the coronal plane, collected in De Olmos cryoprotectant, and stored in well plates at -20 C until staining. For staining, every other brain section was removed from cryoprotectant and placed in phosphate buffered saline with gelatin and triton (PBS-GT; 0.1% gelatin, 0.3% Triton X-100, in PBS). Unless noted, all rinses were done in PBS-GT, all solutions were made in PBS-GT, and all steps took place on a Rotomixer. Tissue was rinsed 4 times for 5 min each to remove cryoprotectant, placed in .1% sodium borohydride for 15 min, and rinsed 4 times until bubbles were gone. Tissue was then blocked in 10% normal horse serum (Vector Laboratories product S-2000) with avidin (1 drop/mL solution; avidin-biotin

blocking kit from Vector Laboratories, product SP-2001) for 1 hour and rinsed 3 times. Tissue was then incubated in primary GFAP antibody (Millipore #MAB360 mouse anti-GFAP) containing biotin (1 drop/mL solution) for 1 hour at room temperature on the Rotomixer, followed by overnight incubation at 4 degrees C off the rotomixer. After incubation in primary antibody, the tissue was rinsed 3 times, then incubated in secondary antibody (biotinylated horse anti-mouse secondary, Vector product BA-2001) for one hour, rinsed 3 times in PBS-GT, and incubated in Vector ABC Elite at half the recommended dilution for one hour. After rinsing, sections were reacted in diaminobenzidine (DAB, Sigma Laboratories) in .05M Tris buffer. Tissue was rinsed and mounted on gelatin-subbed slides out of fresh PBS-GT. Slides were dried overnight before being dehydrated through graded alcohols, counterstained with Harris Hematoxylin, put in saturated lithium carbonate as a bluing agent, coverslipped with Permount, and dried and stored until microscope analysis.

Microscopy and Analysis. The borders of the VMH and its subregions were traced on a Zeiss Axioplan II microscope using an Optronics MicroFire digital camera displayed on a monitor. The boundaries of the VMH and its subregions were traced at low magnification using boundaries from the Paxinos and Franklin mouse brain atlas. Astrocytes were counted and traced at high magnification using an oil-immersion 100x lens, using standards established in our laboratory and described in Johnson et al. 2008. StereoInvestigator (version 10) was used to trace the VMH at low magnification. Using the 100x lens, astrocytes were identified by the presence of at least two primary GFAP branches extending from the cell body. The cell bodies of astrocytes were typically smaller and more star shaped than surrounding cell bodies. Neurolucida

(version 10) was used to trace astrocyte processes. Astrocytes were randomly chosen from those previously counted, but were selected for tracing based on certain criteria, including avoiding overlapping astrocytes, avoiding astrocytes near the border of the region, and ensuring there were clear processes to trace. The investigator selecting and tracing astrocytes for analysis was “blind”, unaware of the group membership of the animal. Twelve astrocytes were traced in the VL subregion of the VMH, and another 12 in the DM subregion, from each side of the brain in each animal. The results of tracings for individual astrocytes were collected and averaged across the subregion for each subject. As no differences between hemispheres were observed, measures were then combined across hemispheres and compared using independent t-tests, with N = the number of animals in each group.

Experiment 2

Production of iTfm Animals. The Cre-Lox system can be used to knock out specific genes. The system utilizes a cyclization recombination enzyme (Cre) that targets a highly conserved 34 base pair sequence (lox site) flanking a DNA sequence of interest. Crossing animals with each of these constructs will result in some offspring carrying both the transgenic cre recombinase enzyme and the floxed gene of interest, so that the gene of interest between the lox sites will be recombined and thereby disabled. For these studies, the DNA flanked by lox sites was exon 2 of the *AR* gene encoding the first zinc finger of the AR DNA-binding domain, which is necessary for recognizing androgen response elements. Deleting exon 2 results in a frame-shift mutation that produces a premature stop codon, and therefore a truncated, nonfunctional AR

transcript and protein. The floxed AR animals are described in (De Gendt et al., 2004) and our iTfm model is described in Chen et al., 2014.

Mice carrying this floxed allele were crossed to animals universally expressing cre recombinase (“deleter” mice, Jackson stock #003724). Some offspring from this cross will have both the floxed AR and cre recombinase, resulting in the removal of exon 2 of the AR. When this recombination occurs in a germ cell of females, they can pass the recombined, dysfunctional allele on to future generations. Such females carrying one X chromosome with a recombined, and therefore dysfunctional AR, can be crossed with C57/Bl6 males to produce offspring of four genotypes: WT males (XY), WT females (XX), females carrying one copy of the nonfunctional *AR* allele (carrier females, used for breeders), and males carrying an X chromosome with the recombined, nonfunctional *AR* allele, which recapitulates the phenotype of the classic spontaneous Tfm males, and so we refer to them as “induced Tfm males” (iTfm males). These iTfm males are genetically male (XY), but due to the nonfunctional AR, appear phenotypically female. Like the spontaneously occurring Tfm (sTfm) mice, they have external female genitalia and nipples, and small, undescended testes in the abdominal cavity. The iTfm mice also show no AR immunoreactivity in the brain, which confirms AR knockout (Chen et al., 2014). While Tfm rats have similar T levels to WT males, iTfm mice have low circulating T levels (Chen et al., 2014), as do sTfm mice. Therefore in studies using iTfm mice to examine the role of the AR, it is important to equalize circulating T in the iTfm mice and WT control males. This iTfm model has become of interest due to the ability to compare region- or age-specific knock-out models using this same floxed AR.

Polymerase Chain Reaction (PCR). As described above in Experiment 1, PCR was used to detect the presence of Sry and the WT or recombined AR allele in animals at weaning. Male offspring of pairing iTfm carrier females and WT sires were classified as one of four possible genotypes- WT males (Sry positive and WT AR), WT females (Sry negative and WT AR alleles), carrier females used for breeding (Sry negative and WT/recombined AR alleles), and iTfm males (Sry positive, recombined AR).

Animal Procedures. Animals were housed in plastic cages on a 12:12 LD cycle and had access to water and food pellets (Harlan Teklad) *ad libitum*. Animals were weaned at postnatal day 21 (PN21), ear punched for genotyping using PCR as described above, and then group housed by sex. Experimental animals were gonadectomized and implanted with subcutaneous Silastic capsules at PN60, using 3-4% isoflurane for anesthesia. Once unresponsive, surgical areas were shaved and disinfected with betadine. For WT males, scrotal sacs were shaved and a small incision was made in the midline. Testes were then brought out through the incision and clamped, silk sutures were used to tie off the fat pad and vas deferens, and the testes were removed. For females, areas of both flanks were shaved. Incisions were made through the skin and underlying muscle layer, and the fat pad containing the ovary was revealed. The ovary was located and tied off with surgical silk sutures and removed. Since iTfm animals produce low levels of T and have small, undeveloped testes deep within the abdominal cavity**,** sham castrations were done in these animals. For all procedures, surgical sutures were used to close the muscle wall and surgical staples were used to close the skin layer.

While still under the anesthesia for gonadectomy, animals were implanted with a Silastic capsule that was either empty (blank; B), or filled with free T (Sigma). The capsules were 3.2mm in outer diameter, 1.6mm inner diameter, and had an effective release length of 6mm of T with a total capsule length of 16mm, as 5mm of each end were filled with silicone glue. The blank capsules were capped with glue and left empty. Capsules were implanted subcutaneously; for males and iTfms, this occurred through a separate incision on the dorsal surface, while in females capsules were inserted through one of the incisions made for ovariectomy. Capsule implant openings were closed with surgical staples. All animals were injected with Ketofen for analgesia (.15mL/animal, concentration of 10mg/mL) during the surgery, and again the following day. Animals were monitored for well-being for 14 days after surgery in compliance with animal care guidelines.

Perfusions. At PN90, animals were perfused for brain collection. Animals were injected with an overdose of sodium pentobarbital. Once unresponsive, blood was first collected from the heart for T assays, and then the animals were intracardially perfused with 0.9% saline followed by 30% phosphate buffered formalin. Brains were removed and post fixed in buffered formalin for at least 30 days. Blood samples taken prior to perfusion were placed in heparinized tubes, spun at 3000rpm for 20 minutes, and 125ul of plasma was taken out and stored at -80 C for later analysis. Samples were then sent to the Diagnostic Center for Population and Animal Health at MSU, using Coat-a-Count Total Testosterone kits. Each sample was run in duplicate, and the average of these two was used to later confirm T levels.

Fixed brains were transferred to 20% sucrose solution overnight before microtome sectioning in the coronal plane at 30um. Sections were collected in a low phosphate- gelatin-triton solution (0.01M PO4 containing 0.1% gelatin and 0.3% triton X-100), and 1 out of 2 sections were then mounted on to gelatin-subbed slides. The slides were dried before staining for Nissl substance with thionin as follows: slides were dehydrated and defatted through a series of graded alcohols and xylene, rehydrated in dH2O, stained in thionin solution, differentiated in acetic acid, and dehydrated through a series of graded alcohols. Slides were then coverslipped out of xylene with Permount and allowed to dry.

Microscopy. A Zeiss Axioplan II microscope with an Optronics MicroFire digital camera was used for microscope analysis. StereoInvestigator software (version 10) was used to trace the VMH, count neurons, and trace neuronal somata. The VMH and its subregions were traced at low magnification using boundaries from the Paxinos and Franklin mouse brain atlas and descriptions in Dugger et al. 2007. Neurons were counted and traced at high magnification. Nissl stained neurons were identified by a distinctly stained nucleolus as described and pictured in Morris et al., 2008.

Experiment 3

Animal Procedures. Male, female, and iTfm animals were bred, housed, and genotyped as described in the previous experiments. The animals were gonadectomized at PN60 and perfused for brain collection at PN90.

GFAP Staining and Analysis. Brains were sectioned and stained for GFAP as in experiment 1. After the tissue was stained for GFAP, the borders of the VMH and its subregions were traced, and astrocytes were then counted and traced as described in

experiment 1. The investigator selecting and tracing astrocytes was uninformed about group membership of each specimen.

RESULTS

Experiment 1: Gonadally intact adults

The sex difference in VMH volume reported in rats was also seen in these gonadally intact mice, as males had a greater VMH volume than females, $t(12)=3.57$, $p=.004$. As in rats, the VL and DM subregions accounted for this effect on overall VMH volume in mice (Figure 2). We also found a sex difference in astrocyte number, as males had significantly more astrocytes than females in the VMH, $t(12)=5.05$, $p=.01$; Figure 3.

The VL subregion also showed several sex differences in astrocyte complexity (Figure 3). VL primary branch length was longer in males than females, ($t(12)=2.32$, $p=.038$), had more branch points, $t(12)=2.54$, $p=.026$, and branch endings, $t(12)=2.34$, $p=.038$. In contrast, astrocytes in the DM subregion did not show any significant sex differences in process complexity: no difference in the primary branch length, $t(12)=.51$ $p=.619$, in the number of branch endings, $t(12)=1.49$, $p=.162$, or in the number of branch points, $t(12)=1.92$, $p=.079$; (Data not shown).

Experiment 2: Androgen manipulations

There was a main effect of adult T treatment on VMH volume, $F(1, 61)=9.63$, $p=.003$, $\eta p^2=.004$, but no effect of genotype, $F(2,61)=.484$,$p=.619$, $\eta p^2=.016$ and no interaction, $F(2,61)=.599$, $p=.553$, $\eta p^2=.019$ (Figure 4). The VL and DM subregions of the VMH accounted for these differences (Figure 5), as there were no differences across genotype or hormone treatment in the A or C subregions (Table 1).

There was also a sex difference in the number of VMH neurons, favoring males, which was unaffected by T manipulations, as indicated by a main effect of genotype, $F(2,61)=6.94$, $p=.002$, $\eta p^2=.185$, but no effect of hormone treatment, $F(1,61)=.633$, $p=.429$, $\eta p^2=.010$, or interaction, $F(2,61)=.200$, $p=.819$, $\eta p^2=.007$. The iTfm males had significantly more neurons than females ($p<.05$) and did not differ from WT males (Figure 6).

There was also a main effect of T treatment $F(1,61)=11.09$, $p=.01$, $\eta p^2=.139$ on VMH neuronal soma size, such that animals with T had larger somata that those given blank capsules (Figure 7). While there was no significant interaction of genotype and hormone treatment, $F(2,61)=1.83$, $p=.168$, $\eta p^2=.050$, there was a trend for an effect of genotype, $F(2,61)=2.43$, $p=.09$, $\eta p^2=.066$, which suggested that soma size in iTfm animals may not be responding to adult T. Therefore, we used a t-test to compare iTfm males with or without T, which indicated no significant difference between them, suggesting that functional AR is necessary for T to increase VMH neuronal soma size in mice, $t(27)=.680$, $p=.50$. This result also indicated that including the iTfm group in the overall analysis might obscure sex differences. Therefore, another ANOVA was conducted examining only males and females, which confirmed a main effect of hormone treatment on neuronal soma size $F(1, 42)=11.22$, $p=.002$, and no significant interaction effect of hormone and genotype, $F(1,42)=.807$, $p=.374$, and no significant effect of sex, $F(1,42)=2.92$, $p=.095$.

Experiment 3: Androgen effects on astrocytes

There was a significant effect of genotype on astrocyte number in the VMH $F(2, 43)=6.104$, $p=.01$, $\eta p^2=.221$; Figure 8. Post-hoc tests showed that males and iTfms

were not significantly different from each other, but both groups had significantly more astrocytes than females. There was no effect of T treatment, *F(*1,43)=1.688*, p*=.21, ηp^2=.038, and no interaction between T treatment and genotype, *F(*1, 43) =1.286, *p* =*.287,* ηp^2=.056, on astrocyte number.

There were no significant differences in the number of branch nodes in astrocytes from the DM subregion across genotypes *F*(2, 43)=.004, *p*=.996, ηp^2=.004, or hormone treatment, *F*(1,43)=.523, *p*=.473, ηp^2=.015, and no interaction of the two factors *F*(2,43)=.314, *p*=.732, ηp^2=.029. Nor were there significant differences in the number of astrocytic branch endings across genotype *F*(2, 43)=.017, *p*=.984, ηp^2=.005 or hormone *F*(1, 43)=.180, *p*=.674, ηp^2=.006, and no interaction *F*(2,43)=.376, *p*=.689, ηp^2=.033. As for DM primary branch length, there was no effect of hormone treatment, *F*(1,43)=.00, *p*=.991, ηp^2=.00, and no interaction of hormone and genotype, *F*=.079, *p*=.924, ηp^2=.012. However, there was a nearly significant effect of genotype, *F*(2,43)=3.20, *p*=.051, ηp^2=.098, as males and iTfms had longer DM branch length than females.

In contrast, the VL subregion of the VMH displayed group differences in several measures of astrocyte complexity (Figure 8). While there was no main effect of genotype on the number of VL branch points, *F*(2, 43)=.169, *p*=.845, ηp^2=.026 and no interaction of hormone and genotype, *F*(2, 43)=.493, *p*=.614, ηp^2=.062, there was a significant main effect of hormone treatment, *F*(1, 43)=13.807, *p*=.001, ηp^2=.279. The results for VL branch endings and VL primary branch length followed the same pattern of differences. For VL branch endings, there was no main effect of genotype, *F*(2,43)=.126, *p*=.882, ηp^2=.015, and no interaction of genotype and hormone, *F*(2,

43)=.767, p=.470, ηp^2=.071, but a significant main effect of hormone treatment, F(1, 43)=13.183, p=.001, ηp^2=.260. For VL primary branch length, there was also no main effect of genotype F(2, 43)=.131, p=.877, ηp^2=.003, or interaction effect, F(2, 43)=.971, p=.387, ηp^2=.029. The VL primary branch length showed marginally significant effect of hormone treatment, F(1,43)=4.066, p=.050, ηp^2=.075.

DISCUSSION

The first experiment examined sex differences in VMH regional volume and astrocytes in gonadally intact male and female mice. VMH volume was larger in males than females, conforming to results in the rat VMH (Dugger et al., 2007). While the VL subregion accounted for the sex difference in the VMH volume in rats, both the DM and VL contribute to the sexual dimorphism in mice. The present study also revealed a sex difference in astrocyte number and complexity in the VMH. As regional volume, astrocyte number, and complexity were all greater in males than females, astrocyte number and structure presumably contributed to the overall sex difference in VMH volume.

While previous studies in Tfm rats showed a prominent role of the AR in full masculinization of the VMH, experiment 2 did not indicate such a role in masculinization of the VMH regional volume or neuron number in mice. Rather, greater circulating T led to increased VMH volume regardless of AR status. Neuron number, however, was sexually differentiated regardless of T or AR, because males and iTfms had more VMH neurons than females, and gonadectomy and T administration had no effect on this measure. In contrast, neuronal soma size in the VMH was greater in T-treated animals

of either sex, and this androgen influence requires a functional AR, as this measure was unresponsive to adult T treatment in iTfm mice.

As iTfms lack a functional AR yet show an increase in VMH volume with T treatment, androgens apparently affect this measure through a non-AR mechanism. The most likely alternative candidate is that T is being aromatized to estrogen (E), and acting through one or both estrogen receptors (ERs). E has been shown to affect behaviors related to the VMH, including aggression in males, supporting this possibility. Adult male Tfm mice show less aggressive behavior than WT males, but Tfm mice show levels of aggression similar to that of WT males if supplemented with E (Scordalakes & Rissman, 2004). This result suggests that an increase of available E after T treatment may affect VMH structure and related behaviors. While T could be acting on ERs in the VMH, it is also possible that T could be acting through ERs in other parts of the brain; many brain regions involved in sexually dimorphic behaviors in rodents are demasculinized in Tfm animals (Zuloaga et al., 2008). The effects of T could occur through action in other parts of the brain that may alter behaviors, and this may have an indirect effect on the VMH. Some of these other brain regions have also been shown to be responsive to adult T treatment in adulthood, including the posterodorsal region of the medial amygdala (Morris et al., 2008). In summary, T is likely acting through an ER-mediated mechanism to increase VMH volume in iTfm mice, but could be acting through indirect routes, via other brain regions, or acting directly in the VMH.

Neuron number in the VMH did not differ between males and iTfms with or without T, and was greater than in females, suggesting that the sex difference in VMH neuronal number is independent of AR and circulating T. Alternatively, neuron number

may be masculinized early in development and remain unaltered by circulating adult T levels. The likely mechanism for this effect would be a perinatal surge in T in males that masculizines VMH neuronal number via ERs. There is indirect evidence suggesting that Tfm mice have T levels near normal male levels in the perinatal period when T may have an organization effect on the brain (Goldstein & Wilson, 1972). This result suggests that T via aromatization to E would masculinize the brain early in development in mice, with or without a functional AR.

On the other hand, AR may be necessary for increases in soma size of VMH neurons in response to adult T treatment in mice, because soma size in iTfms did not respond to T treatment. WT male and female mice given T had larger neuronal soma size in the VMH than same-sex controls given blanks. This hypothesis that AR mediates androgens' "activational" effect of increasing VMH soma size could be tested in the future by using animals that have mosaic AR expression, with some cells expressing WT AR and other cells expressing the Tfm allele; such mosaic expression occurs in females carrying one copy of the Tfm allele. If such an experiment found only neurons expressing functional AR increase in size after T treatment, that would suggest a cell-autonomous response to T.

We also examined the role of circulating T on VMH astrocytes in males, females, and iTfms. Again, male mice had more astrocytes than females. Adult T treatment had no effect on this measure. Importantly, iTfm mice were equivalent to males, so masculinization of astrocyte number is also independent of AR. As for complexity of astrocyte processes, there were more astrocytic process endings and branch point in animals treated with T than those given blank capsules in the VL subregion. This was

true in both sexes and also in iTfm males, so T's effects on complexity of astrocytes in the VL are independent of AR as well. There were no effects of T treatment on astrocyte complexity in the DM subregion, which suggests that astrocytes of the VL subregion are specifically responsive to T, while those in the DM subregion are not.

Since astrocytes play a variety of roles in synaptic transmission, the effect of more, or more complex, astrocytes may be related to these functions. As increased astrocyte branch complexity has been associated with increases in functional synapses (Elmariah et al., 2004; Pfrieger & Barres, 1997), and branch processes may help form new synapses and regulate contact between neurons (Hatton, 2002; Nishida & Okabe, 2007; Ullian et al., 2001), increased astrocyte complexity in males may lead to greater plasticity in the VL portion of the VMH. The VMH is involved with several sexually differentiated behaviors, including female-biased behaviors, which may seem conflicting in light of males having more, and more complex, astrocytes. However, these synapses or functions could also be inhibitory in nature. The mouse VMH VL subregion is involved with male aggression, and there is a separate, distinct set of neurons in this region involved with mating behaviors; the neurons involved with mating are inhibited during aggression (Lin et al., 2011). Future studies are needed to understand whether and how these astrocytic differences affect sexually differentiated behaviors related to the VMH.

Overall, these results demonstrate that the VMH is a highly sexually dimorphic region in mice. Males had a larger VMH, with more neurons and more astrocytes than females, and males also had more complex astrocytes than females in the VL subregion. Some sexually dimorphic aspects of the mouse VMH are affected by levels of circulating androgen and so may reflect the normal sex difference in hormone levels.

The volume of the VMH increases with adult T treatment in both sexes, and also in iTfm mice with dysfunctional AR. The DM and VL subregions seem to account for this effect on overall VMH volume. Neuronal soma size is also larger with T treatment in adulthood, although iTfms show no such response, suggesting a role of the AR in this effect. Finally, neuron number is higher in males and iTfms than females, regardless of adult T treatment, suggesting a sex difference that is independent of both the AR and adult circulating androgens. T treatment increased astrocyte complexity in the VL subregion of the VMH in all three groups, so this sex difference appears to be an activational response to circulating androgens, which is independent of AR. Together, these studies demonstrate that the VMH is sexually dimorphic in mice, and that the region is responsive to adult T treatment. As the VMH is related to several sexually dimorphic behaviors, future studies can clarify how these structural dimorphisms may relate to sex differences in behavior in mice.

CHAPTER 3: DISCUSSION

The results of this dissertation showed that the ventromedial nucleus of the hypothalamus (VMH) is sexually differentiated in mice, including in measures such as the overall VMH volume, volume of specific subregions, neuronal number and soma size, and astrocytic measures. Circulating levels of adult testosterone (T) and functional androgen receptor (AR) played various roles in the sex differences observed, as further discussed below. This final chapter synthesizes and discusses results from this dissertation and future experiments that could help further understanding of the function and hormonal responsiveness of the VMH.

The Mouse VMH is Sexually Dimorphic

This dissertation centered around the question of whether the VMH is sexually differentiated in mice as it is in rats. In rats, the VMH is larger in males than females, with the ventrolateral (VL) subregion accounting for most of the difference in overall volume (Dugger et al., 2007), but it was unclear if this difference was also present in mice. First, we examined VMH volumetric sex differences in unaltered WT males and females to see if a sex difference could be detected, and the results showed that WT males indeed have a greater VMH volume than WT females, with the DM and VL subregions accounting for this difference. Examining wildtype (WT) males, WT females, and induced testicular feminization (iTfm) males lacking a functional AR, with and without adult T, showed that circulating T levels increased VMH volume in the dorsomedial (DM) and ventrolateral (VL) subregions in all three groups, even in iTfms lacking functional AR. These results indicated that the sex difference in VMH morphology is dependent on circulating T levels. Therefore, it could be hypothesized

that higher levels of circulating T in WT males contributes to the overall sex difference, as females have low levels of circulating T and respond to adult T treatment. The results of this experiment also demonstrated that males have more VMH neurons than females, and that iTfms do not differ from WT males in this regard, suggesting a sex difference in neuron number that is independent from either adult circulating T levels or AR status. These studies showed that VMH volume and neuron number are sexually differentiated in the mouse brain, with the sex difference on volume dependent on circulating T while the sexual dimorphism in neuron number is not.

Experiment 1 also showed that astrocytes in the VMH are sexually differentiated, as WT males have more astrocytes and more complex astrocytes than WT females; the VL subregion's astrocytes had longer primary branch length, more branch points, and more branch endings in males than females. When looking at males, females, and iTfms with and without T, I saw a significant effect of genotype, with males and iTfms not significantly differing from each other, but both having more astrocytes than females; these measures did not respond to manipulations of circulating T. These results indicate that astrocyte number and complexity are sexually differentiated in the mouse VMH, and thus, it is likely that astrocytes at least partially contribute to the overall sex difference in VMH volume.

Together, these results show that the mouse VMH is indeed sexually differentiated in several morphological measures, including volume, neuron number, astrocyte number, and astrocyte complexity. While some observed measures depended on circulating adult T (described further below), others, such as neuron number and astrocyte number, persisted independent of adult circulating T levels or AR.

Presumably these sex differences are established early in life. Some astrocyte sex differences required follow-up studies to see if they are dependent on circulating T, described next.

The Mouse VMH Responds to Adult Circulating T

In addition to the sex differences discussed above, these experiments also showed that various elements of VMH morphology respond to adult circulating T. I observed that circulating T led to greater VMH volume in males, females, and iTfm males. Specifically, the DM and VL subregions of the VMH increased in volume with T treatment, and the volume of these two subregions accounted for the overall effects of circulating T on volume. As this effect was also seen in iTfm males lacking functional AR, T is acting through an AR-independent mechanism to have this effect on VMH volume. It seems likely then, that these results are due to T providing ligands to activate estrogen receptors (ERs) to alter VMH regional volume. The results also showed that soma size increased with adult T-treatment in males and females compared to those without T treatment. However, this effect was not seen in iTfm animals, suggesting that a functional AR is necessary for T to affect neuronal soma size in the VMH.

In the last experiment, I found that astrocytic complexity measures responded to circulating adult T, as T-treated animals had more complex astrocytes than those without T. In the VL subregion, complexity measures of branch endings and branch points and branch length were greater in males, females, and iTfms treated with T, regardless of AR phenotype or genotype. As these effects were observed even in animals without a functional AR, T is apparently acting through an AR-independent mechanism, again likely ER-mediated processes triggered by aromatized metabolites of

T. Additional experiments, discussed later, could further clarify the role of AR and circulating androgens in sex differences in the VMH.

Discussion of Results

The VMH is an area of great interest to the study of sex differences and the role of steroid receptors in the brain and behavior. The VMH is associated with several sexually differentiated behaviors, including sexual behaviors, aggression, and others (Kow & Pfaff, 1998; Lin et al., 2011; T. Matsumoto & Yamanouchi, 2000; Pfaff & Sakuma, 1979; Yahr & Greene, 1992). The region expresses high levels of steroid receptors, especially in the VL and DM subregions (Simerly et al., 1990). The experiments in this dissertation further investigated sexual dimorphisms in VMH structure, and found that the area is sexually dimorphic and responsive to adult T treatment in mice, with many of these effects observed in the DM and VL subregions of the VMH, which are associated with sexually differentiated behaviors. While some of these differences were previously studied in rats, the results were slightly different in mice; in rats, only the VL was sexually dimorphic in volume, but in mice both the DM and VL regions were sexually dimorphic. These results suggest that the sex difference in VMH volume is slightly different in mice than rats, but still focused on the subregions associated with sexually differentiated behaviors. However, astrocytes in the mouse VL subregion and not the DM subregion were sexually dimorphic and more responsive to circulating T, which is in line with the volume differences restricted to the VL subregion of rats.

This dissertation also examined the role of AR and circulating hormones in these differences, and found that many of the sex differences depended on adult circulating T,

sometimes even in animals without a functional AR, suggesting that T is acting through an AR-independent mechanism. This is different than our previous study in rats, which found VMH volume in Tfm rats was intermediate between that of males and females, suggesting that a functional AR was required for full masculinization of the region. As discussed in the introduction, Tfm mice and rats have different levels of circulating T and AR functionality, which may account for some of these observed differences (Zuloaga et al., 2008). Additionally, rats and mice are of course different, and are not necessarily expected to share all similarities, and other sex differences in the brain also differ between mice and rats.

Overall, it seems that the mouse VMH is more responsive to adult T, and less dependent on AR. This may be because Tfm rats have higher levels of circulating T and more residual AR functionality than Tfm mice; Rats may have some residual action of T on AR or ER, whereas mice have less, if any, without supplementing T levels. As rats have higher levels of T that can be aromatized, even if being aromatized at lower levels than in WT males, this could explain why VMH morphology and associated behaviors tend to be more intermediate between WT males and females in Tfm rats as compared to Tfm mice. Tfm rats have near-normal T levels throughout adulthood, and more residual AR functionality, while Tfm mice have lower levels of T and low AR activity. Presumably, lack of T and AR activity through the lifespan, including the pubertal period, could affect these differences between mice and rats, both within the VMH and in other areas of the brain.

Given the observed differences between mice and rats in regards to VMH morphology, residual AR functionality, and T levels, it is also important to consider if and

how these results relate to observed behavioral differences in the literature. The anatomical results of the VMH and T levels/AR functionality of Tfm mice and rats line up with some previous behavioral findings from other studies, although not with all. Some behaviors follow the pattern of being more intermediate in Tfm rats, and feminine in Tfm mice until they receive supplemental T or E. For example, Tfm rats show decreased masculine mating behaviors compared to WT males suggesting a role of the AR in masculinization of this behavior in rats (Beach & Buehler, 1977; K.L. Olsen & Whalen, 1981; Olsen, 1992; Shapiro et al., 1976; Zuloaga et al., 2008). Tfm mice also show reduced masculine sexual behavior compared to WT males on measures such as mounts and thrusts; however, this difference is eliminated if the Tfm mice are treated with E (Bodo & Rissman, 2007; Ohno, Geller, & Lai, 1974). As with the results observed in this dissertation, these previous findings show a role of AR in rats, but a limited role of AR during development in mice, as E treatment is sufficient to masculinize this behavior. However, other studies show a partial role of AR in Tfm mice on measures such as partner preference and exploring male-soil bedding where they behave similar to WT females; these differences are not eliminated with E-treatment. As mice lack functional AR throughout development, the question remains when functional AR is necessary to masculinize this behavior.

Additionally, aggression has been studied in Tfm rats and mice with the results showing varying roles of the AR and circulating hormones. Studies in rats show that Tfm males display less play fighting than WT males, and do not show the decreased levels of play fighting as they age that males typically show (Field, Whishaw, Pellis, & Watson, 2006; Meaney, Stewart, Poulin, & McEwen, 1983; Meaney, 1988). However, Tfm rats

do show masculine declining levels of some aspects of play fighting, suggesting that AR is involved in some aspects of juvenile aggressive behavior (Field et al., 2006). In mice, however, there is a more limited role of AR. Tfm mice show decreased aggressive behavior, but this difference goes away when supplemented with E. Studies with knockout ER animals suggest that aggression may be primarily mediated through ERs in mice (Scordalakes & Rissman, 2004). Again, this pattern of results follows closely with the results of this study in that Tfm rats tend to indicate more of a role of the AR in sexually dimorphic behaviors than Tfm mice.

As the VMH is related to several sexually dimorphic behaviors, including aggression and mating, it is also important to examine the relationship of these networks within the VMH. Traditionally, the VMH was associated with female mating behaviors, but its known role has expended. More recently, an optogenetic study identified the VL subregion of the VMH as being a locus of aggressive behavior in males (Lin et al., 2011). Optogenetic stimulation of VL neurons elicited attack behavior in males against females and males. In addition to demonstrating the role of the VL in aggressive behavior, this study also shed important insight on how the networks for aggressive behavior and sexual behavior may interact; the neurons stimulated during attack were inhibited during mating (Lin et al., 2011). This finding suggests that the neural networks involved with aggression and mating may be in competition with each other, which means that the networks for these social behaviors coexist in the brain, and are overlapping, but distinct, subpopulations of neurons. This study shed further light on the role of the VMH in males, and also demonstrates that the networks for these distinct social behaviors exist separately and in competition within the VMH.

It is also important to consider how T via aromatization to E affects VMH morphology, and optogenetic studies provide evidence that it is estrogen receptor-expressing neuron in males that can lead to mating and aggression specific behaviors. In male mice, stimulating neurons with the estrogen receptor in the VL subregion was sufficient, and necessary, to induce mounting and aggressive behavior; stimulating neurons without the ER was not sufficient to elicit this behavior (Lee et al., 2014). Of interest, the behavior induced by stimulating the ER+ neurons occurred in a gradient; a low-level stimulation produced mounting behavior, while increased stimulation led to male aggressive behavior. The same stimulation in the VL of females included some mounting behavior and social investigation, but not attack; as females did not show aggressive behavior in this paradigm as males did, these results indicate that sex differences in aggression might be occurring within or downstream of the VL (Lee et al., 2014).

Additionally, it is important to note that the VMH is connected to other brain regions that are also involved in sexually dimorphic behaviors. Of note, the VMH has afferent connections from the preoptic area and the amygdala (Canteras, Simerly, & Swanson, 1994; Fahrbach, Morrell, & Pfaff, 1989; McClellan, Parker, & Tobet, 2006), and has efferent connections to the amygdala, medial preoptic area, the bed nucleus of the stria terminalis (Saper, Swanson, & Cowan, 1976), among others. These regions are involved with several sexually dimorphic behaviors as reviewed in Zuloaga et al., 2008. Thus, the VMH is highly connected to a network of brain regions involved in sexually dimorphic behaviors, and effects of circulating T on other brain regions could affect behaviors related to the VMH. Of interest, many of the afferent connections to the VMH

go to the VL subregion (Fahrbach et al., 1989), which is the region containing steroid receptors that is larger in males than females in mice and rats, intermediate to males and females in Tfm rats, and T responsive in adult males. Additionally, in the guinea pig, many of the VMH sites containing ERs project to other areas with ERs (Turcotte & Blaustein, 1999). Therefore, it is unclear whether circulating T is acting via ERs within the VMH, acting in other brain regions and having downstream effects on VMH morphology, or, a combination of these. As discussed above, results of an optogenetic study in the mouse VMH leave open a role of other brain regions in aggressive behavior (Lee et al., 2014).

Many of the present results in mice suggest that T is acting through an AR-independent mechanism, and the likely scenario is that T is being aromatized to estrogen (E), and acting through one or both estrogen receptors (ERs). Other studies have shown that E acting through ERs affects behaviors that are associated with the VMH, including aggression in males (Scordalakes & Rissman, 2004), which supports the notion that E is a possible mechanism for the observed effects. Scorkalakes & Rissman (2004) reported that adult Tfm mice given E show levels of aggression that are similar to those of WT males; giving T would lead to an increase of available E, which may have an effect on the structure of the VMH or the related behaviors. T and its metabolites could be acting on ERs in the VMH, or, it is also possible that T or E is acting through ERs in other parts of the brain, which then affect the VMH. The effects of T could occur through action in other parts of the brain that may alter behaviors, and this may have an indirect effect on the VMH. Some of these other brain regions have also been shown to be responsive to adult T treatment in adulthood, including the posterodorsal region of the

medial amygdala (Morris, Jordan, & Breedlove, 2008), and others (reviewed in Zuloaga et al., 2008).

Since males, females, and iTfms are showing changes in VMH morphology due to manipulations of T, many of the dimorphisms studied in this dissertation may not be organized in development, but rather respond to adult levels of circulating hormones. Further experiments, discussed next, could help differentiate which of these observed sex differences, if any, are organized at some point in development, either perinatally or in puberty. Sex differences in neuron number and astrocyte number were observed in this study, which may be organized in development, as they did not differ between males and iTfms with or without adult T treatment. Furthermore, both of these groups had more neurons and astrocytes than did females, suggesting that this sex difference is independent of both adult circulating T and AR. The likely mechanism for this result would be a perinatal surge in T in males that masculizines the number of VMH neurons and astrocytes via ERs. However, further experiments would be needed to examine if or how these sex differences are organized in development.

Future Directions

I examined the role of AR and circulating T in sex differences in the VMH, and future studies could further these findings in several ways. First, the iTFM mice in these experiments lacked functional AR throughout development; while we supplemented adult T to equalize levels, iTFM mice lacked circulating T before this time, including during puberty. There is indirect evidence that iTfm mice have the same perinatal T surge as WT males, which is when T may have an organizational effect on the brain (Goldstein & Wilson, 1972), but T could be given to perinatal animals, including females,

to see how it affects adult VMH morphology. Future experiments could also implant T prior to puberty to look for effects on VMH morphology in iTfm males. As VMH volume increases with adult circulating T in iTfms and shrinks in WT males with T removed, I would not predict that pre-pubertal T would have a lasting or permanent impact on this measure. However, a study could determine if pre-pubertal T would lead to changes in morphology of other measures, such as astrocyte complexity, in iTfms lacking functional AR and/or in WT females. Alternatively, WT males could be castrated prior to puberty to see what effect this has on adult VMH measures. While adult VMH morphology has been studied in rats and mice, it is unclear if this sex difference exists before puberty or how it changes throughout development; studies could also ask whether WT males and females show sex differences in VMH volume, neuron number, soma size, or astrocytes before puberty.

Additionally, this study could also be extended to rats to see what astrocyte number and complexity are like in males, females, and Tfms. While VMH volume has been studies in rats, astrocytes have not been examined in this way. By comparing WT males and females, this study would inform as to whether there was a sex difference in these measures. Additionally, this study would also address if there was a role of AR in astrocyte number or complexity. It could be predicted that, mirroring VMH volume results, Tfm rats might have astrocyte numbers and/or measures of complexity intermediate to those of males and females. Such a study could elucidate if astrocyte numbers and complexity measures follow the same pattern as VMH volume results. As Tfm rats have normal levels of circulating T, hormone manipulations wouldn't be

necessary; however, gonadectomizing and implanting with E could help parse out a potential role of AR or ER on observed differences in this paradigm.

Future studies could also ask about the role of E on astrocytes in the mouse VMH. One question is whether astrocyte complexity measures change through the estrus cycle. Based on the results of this experiment, it could be hypothesized that astrocytes might be more complex when E levels are higher, such as in diestrus and proestrus. The results of this study would show how quickly these changes might occur in response to E levels. As there are other hormonal factors such as progesterone that might have an impact in the previous paradigm, one could also ovariectomize females and implant with E or a B capsule to see what impact E alone has on astrocytic measures. A time-course experiment could be done, with animals being sacrificed at different intervals to see how soon astrocyte complexity changes.

To further understand the role of circulating androgens and the AR, mice with mosaic expression of the AR could be studied. Since the AR gene is located on the X chromosome, it is possible to utilize female mice that are heterozygous for the Tfm mutation, meaning, they carry one normal copy of the AR gene and one with a Tfm mutation (Watson, Freeman, & Breedlove, 2001; Yarbrough et al., 1990). Due to random X gene inactivation in females, only one copy of the X chromosome gets expressed in each cell, which in this case would result in mosaic expression of the AR gene by cell. I showed that soma size was larger in males and females with adult T compared to those without, but this effect was not seen in iTfm males. Experiments using mosaic animals could ask whether soma size is enlarged by adult T treatment only in VMH neurons with a functional AR, and not those with the iTfm mutation. If so,

that would indicate androgens act directly upon VMH neurons to increase their soma size. If T treatment affects soma size in both WT and iTfm neurons of the VMH, then this could not be a cell-autonomous response.

Another future direction includes examining whether astrocytes in the mouse brain require steroid receptors to respond to T. Many studies show that astrocytes express steroid receptors (reviewed in Garcia-Segura & Melcangi, 2006), and we could further examine whether astrocytes in the mouse VMH express AR and/or ER, and if either of these receptors are necessary for astrocytes to respond to circulating T levels as observed in the current studies. One could also use animals with ERs knocked-out in astrocytes to see if E is acting directly on astrocytes to impact morphology. In a previous study using astrocyte-specific ER knock-out, it was shown that steroid hormones may be able to act directly on ER on astrocytes. Future studies could utilize this type of knock-out to see if the results of this dissertation depended on ER on astrocytes, or, if E is acting on ERs elsewhere.

Of course, the question remains, what is the functional significance of these structural differences in the VMH? What does having a larger VMH, or more complex astrocytes in the VMH, mean in regards to brain function or behavior? The answers to these questions are not presently clear, but can be hypothesized based on previous studies, and then tested. Astrocytic complexity has been associated with increases in synapse density and synapse number (Elmariah et al., 2004; Pfrieger & Barres, 1997), and decreases in dendritic spines (Mong et al., 2001), so it could be hypothesized that something in the network of the VMH, and possibly with associated brain regions, is changing in response to adult levels of circulating T, presumably in response to its

aromatized metabolite E. Increases in synapse density and number would potentially signal increased network function or efficiency, while decreases in dendritic spines might suggest the opposite; future examination of these measures would help clarify if there are sex differences in what is changing in the VMH or other areas in response to levels of circulating hormones. These changes in astrocyte complexity could be enhancing networks of related behaviors or functions, including potentially on an inhibitory network; however future studies would be needed to clarify the mechanisms by which this was happening.

Due to the emergence of some newer genetic technologies, including Cre-Lox and optogenetics, it is possible to test some of these questions in a more direct fashion. CreLox was used to create our iTfm animals that universally lacked AR expression, and CreLox genetic tools also allow for more nuanced gene deletions, such as targeted site-specific deletion of certain genes under the control of specific promoters. Of interest to this dissertation, site-specific deletion means that AR could be selectively deactivated in certain cell-types to study what impacts this has on sex differences in the brain or behavior. If AR is knocked-out in neurons or in astrocytes, what impact does circulating T have on AR volume, neuronal soma size, astrocyte number, or astrocyte complexity? Additionally, optogenetic tools allow activity to be effectively turned “on” or “off” in certain brain regions. Further experiments could use this method to further understand the role of VMH neurons and astrocytes. Other studies use optogenetics to selectively increase astrocyte activity and observe effects on behavior, including several studies in the hypothalamus (Li, Agulhon, Schmidt, Oheim, & Ropert, 2013; Pelluru, Konadhode, Bhat, & Shiromani, 2016; Perea, Yang, Boyden, & Sur, 2014). Experiments could be

designed that selectively activate astrocytes in this region and see what impact this has on sexually differentiated behaviors.

Summary

The experiments in this dissertation probed sex differences in the mouse VMH. I found that many aspects of VMH morphology are sexually differentiated and/or dependent on adult levels of circulating androgen hormones in mice. Specifically, I found that VMH volume was sexually differentiated and this sex difference was dependent on circulating T acting through an AR-independent mechanism. I also showed that neuron number and astrocyte number are greater in males and iTfms than females, independent of circulating T, which also indicates this sex difference is independent of AR as well. On the other hand, the size of neuronal somata in the VMH was increased by exogenous T only in WT males and females, not iTfm males, indicating that this effect of the hormone is mediated by AR. Finally, I found that astrocytes were more complex in the VL subregion of the VMH in males than in females, and that complexity was greater in males, females, and iTfms given adult T. Taken together, these findings of sex differences and hormone responsiveness in the mouse VMH provide potential explanations for the robust sex differences in the behaviors associated with this brain region.

APPENDIX

Table 1.
Analysis of male, female, and iTfm mice with or without adult T treatment showed no differences in the volume of VMH A or VMH C by hormone treatment or genotype; all *p* >.05. The volume is measured in squared microns.

Means ± SE	**WT M + T**	**WT F + T**	**iTfm + T**	**WT M + B**	**WT F + B**	**iTfm + B**
VMH A (um^3 x 10^6)	3.78 ± .69	3.87 ± .69	3.065 ± .62	4.10 ± .65	2.74 ± .62	3.86 ± .73
VMH C (um^3 x 10^7)	3.83 ± 1.90	3.68 ± 1.90	4.09 ± 1.81	3.86 ± 1.90	3.73 ± 1.74	3.55 ± 2.00

Figure 1.
The ventromedial nucleus of the hypothalamus (VMH), including the ventromedial subregion (VL), central subregion (C), and dorsomedial subregion (DM), is pictured; the anterior subregion is not present in this section. The tissue was sectioned at 30um and Nissl-stained with thionin.

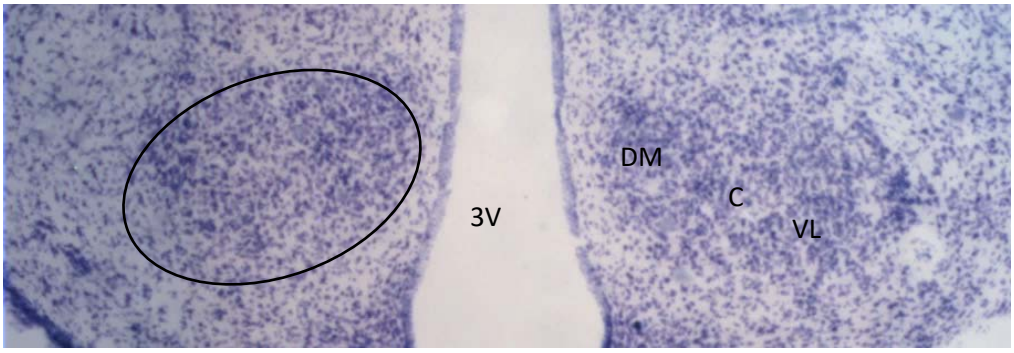


Figure 2.
The total volume of the ventromedial hypothalamus (VMH) is sexually differentiated in mice, as it is larger in wildtype males than wildtype females (A). This sex difference is accounted for by the ventrolateral (VL) (B) and dorsomedial (DM) (C) subregions, which are larger in males than females. Volume of the anterior and central subregions did not differ between males and females (data not shown).

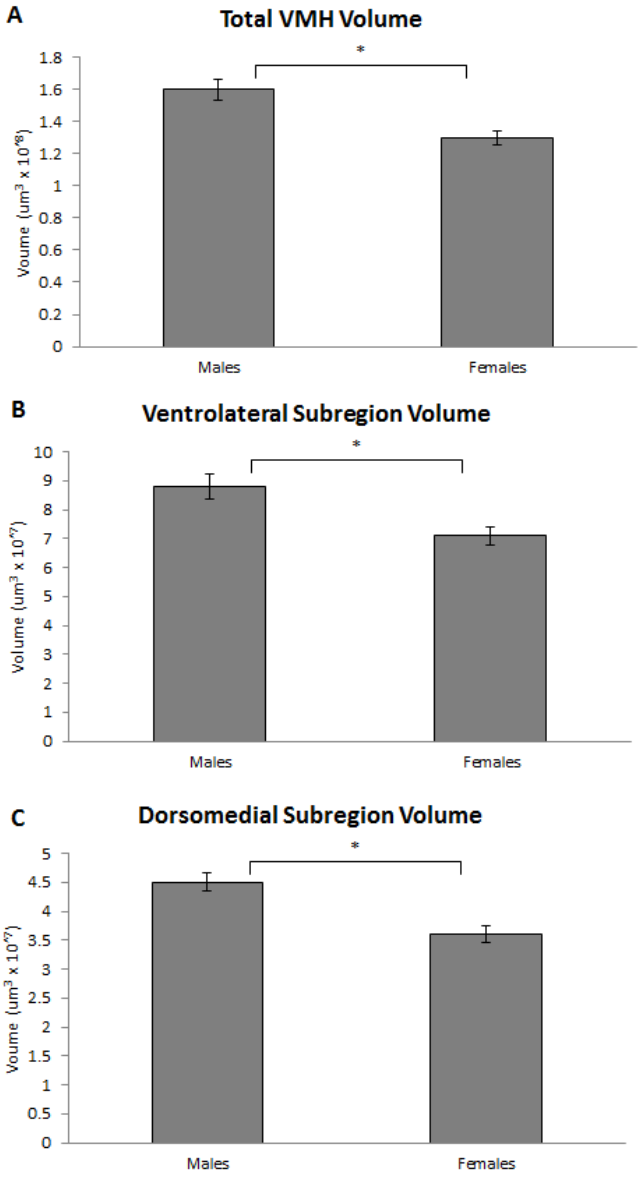


Figure 3.
A) Astrocyte number is sexually differentiated in the ventromedial hypothalamus (VMH) of mice, with wildtype males having more astrocytes than females. Astrocyte complexity is also sexually differentiated in the VL subregion of the VMH, as males' astrocytes have more branch points (B), more branch endings (C), and longer branch length (D; measured in microns) than those of females. There was no sex difference in astrocyte complexity in the DM subregion. As the volume of VMH is also greater in males than females, astrocytes presumably contribute to the overall sex difference in volume.

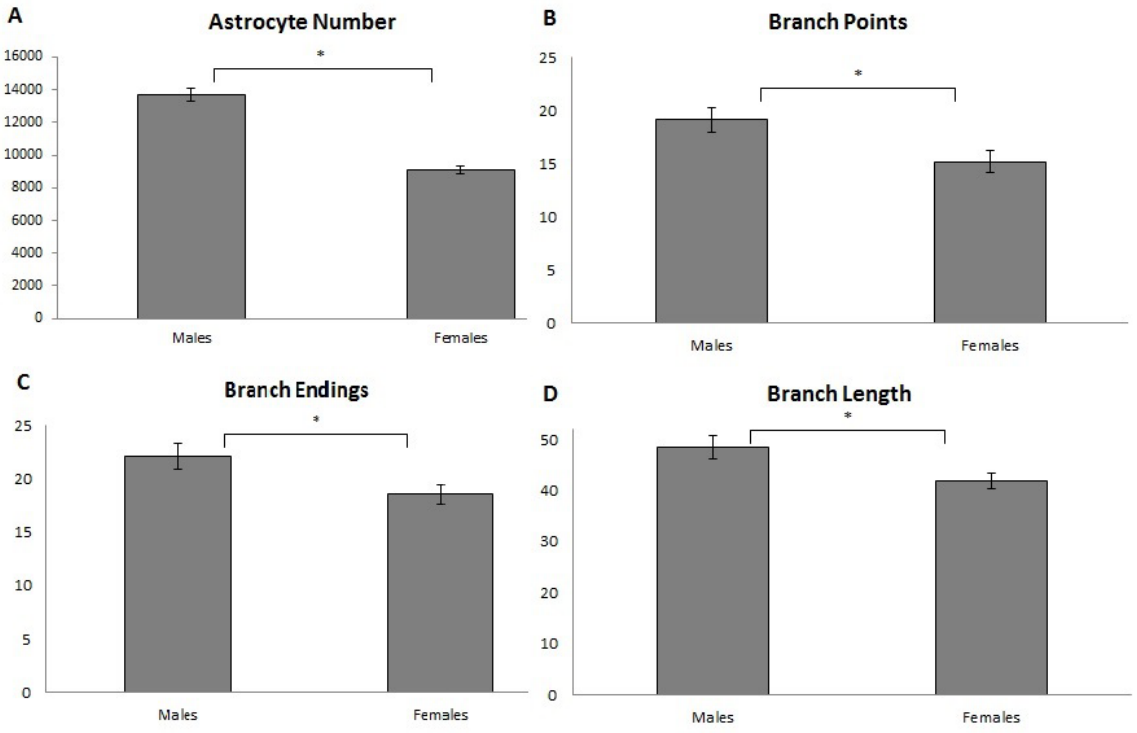


Figure 4.
Total VMH volume was greater in gonadectomized animals treated with T than those given blank (B) capsules in males and females, and also in iTfm mice, which have no functional androgen receptors (ARs). These results suggest that T is acts through an AR-independent mechanism to increase VMH volume in adult mice.

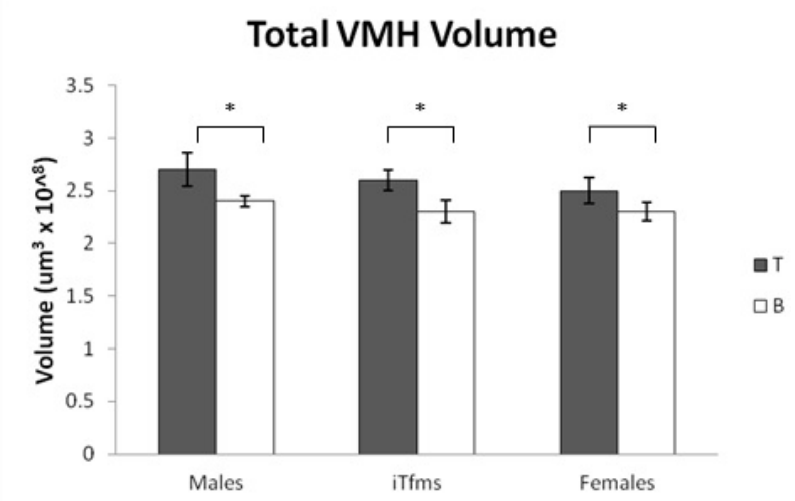


Figure 5.
Volume of the DM (A) and VL (B) subregions of the VMH was greater in T-treated animals than animals given blank (B) capsules, and there was no effect of genotype on these measures. The volume differences in the DM and VL subregions accounted for differences in the overall VMH volume, as volume in the A and C subregions did not show any significant differences by hormone treatment or genotype (Table 1).

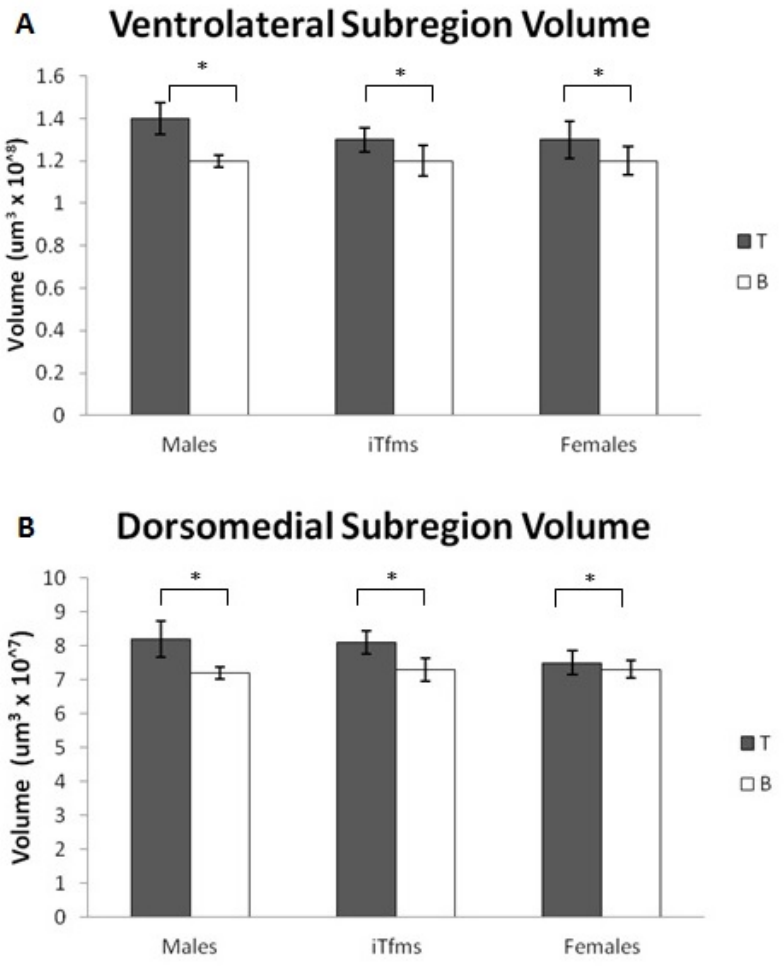


Figure 6.
VMH neuron number was greater in males and iTfms than females, and there was no effect of adult T-treatment on this measure. These results suggest that this sexual dimorphism in neuron number is established early in life through a mechanism that is independent of AR.

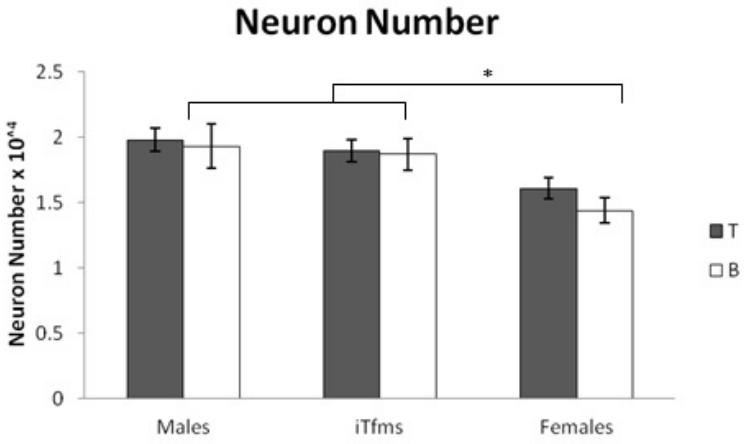


Figure 7.
Analysis of male, female, and iTfm mice with or without adult T treatment showed that neuronal soma size was larger in T-treated animals (main effect of T, *p*= .01). There was no significant interaction, but a marginal effect of genotype prompted independent t-tests in each group, which revealed that VMH soma size was larger in the T-treated animals only in males and females, not in iTfm mice, which carry a dysfunctional androgen receptor (AR). These results indicate that a functional AR is necessary for circulating T to increase neuronal soma size in the VMH.

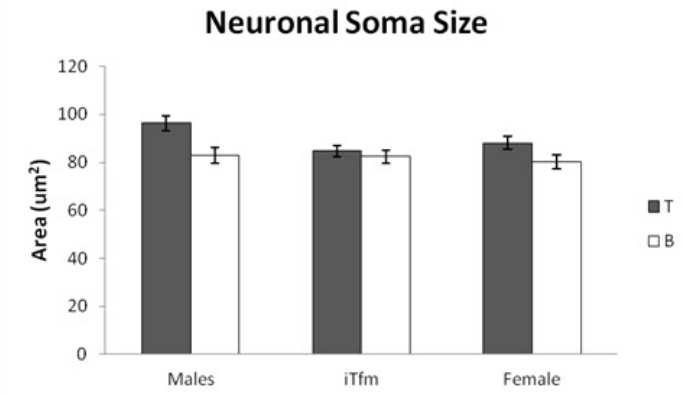
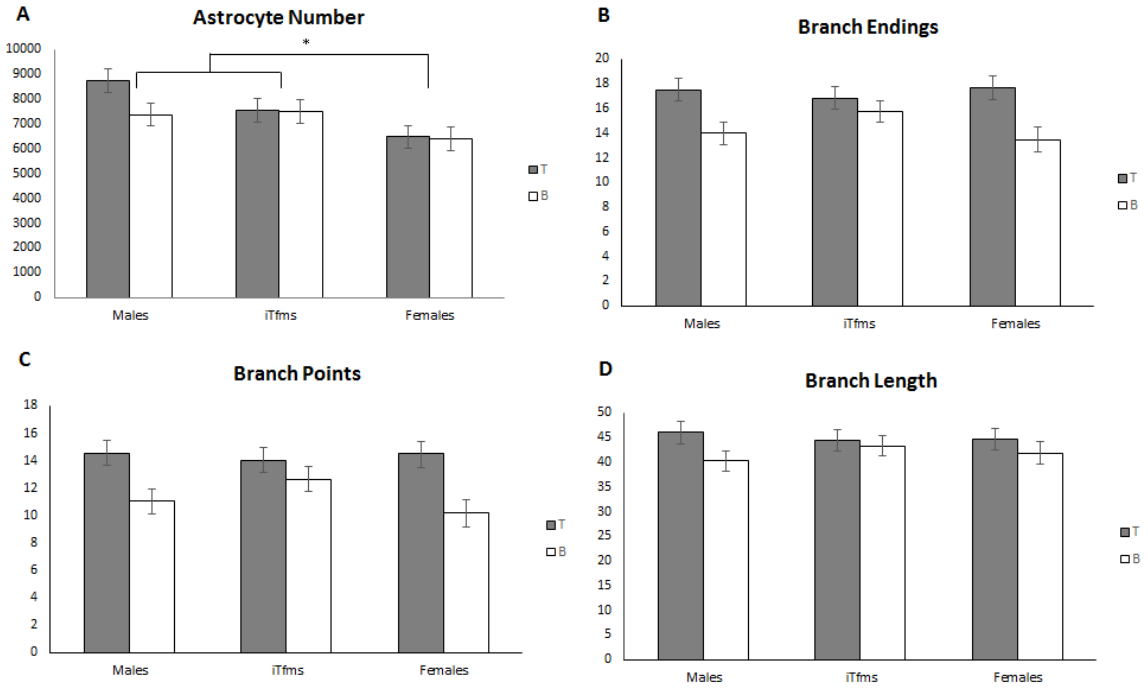


Figure 8.
Astrocyte number did not significantly differ between males and androgen receptor deficient induced testicular feminization (iTfms) mice, but both had significantly more astrocytes than did females (A). There was no significant effect of adult T treatment on astrocyte number, nor any interaction of genotype and hormone treatment, for that measure. Astrocytes were more complex in males, females, and iTfms with adult T compared to those without T. T-treated animals had more branch points (B), more branch endings (C), and longer branch length (D; measured in microns) than controls in the VL subregion of the VMH. There was no significant effect of genotype or interaction of hormone treatment and genotype for astrocyte complexity. There were no significant group differences in astrocyte complexity in the DM subregion of the VMH.



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