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Molecular mechanisms underlying sexual differentiation of the brain and behavior

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Molecular mechanisms underlying sexual differentiation of the brain and behavior

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Doctor of Philosophy in Human Genetics

By

Tuck Cheong Ngun

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ABSTRACT OF THE DISSERTATION

Molecular mechanisms underlying sexual differentiation of the brain and behavior

By

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Doctor of Philosophy in Human Genetics

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Professor Eric Vilain, Chair

The brains of males and females are different anatomically and chemically. There are also sex differences in neurological disease, cognition and behavior that are presumed to be downstream consequences. Two main factors have been implicated in sexual differentiation of the brain: gonadal hormones and direct genetic effects. Here, we explore the role of sex chromosomes in the brain and behavior and the molecular mechanisms mediating the effects of these factors.

We investigated the contribution of sex chromosomes to sex differences in brain and behavior by studying a novel mouse model of Klinefelter Syndrome (KS) termed the Sex Chromosome Trisomy (SCT) model. KS is characterized by the presence of an additional X chromosome in men. We investigated the extent of feminization in XXY male mice. We found that partner preference in XXY males is feminized and that these

differences are likely due to interactions of the additional X chromosome with the Y. We also found that expression of a small but highly significant proportion of genes is feminized in the bed nucleus of the stria terminalis/preoptic area (BNST/POA) of XXY males, which represent strong candidates for dissecting the molecular pathways responsible for KS-specific phenotypes.

We also investigated whether DNA methylation could be one of the molecular mechanisms that mediate the long-lasting, irreversible effects of perinatal testosterone in the BNST/POA. Using a genome-wide approach, we found that methylation at 45 genes was affected three days after the exposure. This number ballooned to 740 in adult animals. There was also a shift to a more masculine pattern of DNA methylation during adulthood in females that had seen perinatal testosterone. These results strongly suggest that perinatal testosterone confers an initial imprint that is amplified over postnatal development. We also observed sex differences in methylation at numerous genes.

The interplay between gonadal hormones and sex chromosomes is a complex one. Collectively, our results provide further support for the theory of direct genetic effects in brain sexual differentiation and suggest that DNA methylation may be one mechanism that mediates not only the effects of gonadal hormones but also direct genetic effects.

The dissertation of Tuck Cheong Ngun is approved.

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2012

DEDICATION

For Dayle, who believed in me even when I didn't believe in myself.

You always made sure I was nothing less than fabulous.

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ACKNOWLEDGEMENTS

The work presented in Chapters 2 and 3 of this dissertation are based on data from the bed nucleus of the stria terminalis/preoptic area (BNST/POA). However, I would like to take this opportunity to clarify that the work in those chapters are part of a larger project that also included investigation of the striatum by Negar Ghahramani. All data pertaining solely to the striatum have not been included.

Firstly, I would first like to acknowledge my mentor, Dr. Eric Vilain, for his guidance, support and tireless energy. Even when things looked their bleakest, he never gave up and was always certain we'd find a way through. I have learned much from Eric, not just about how to be a better scientist but also about how to be a better person. I would also like to offer innumerable thanks to my close friend and collaborator, Negar Ghahramani. This work would have been impossible without her. I could not have wished for a better partner in this endeavor. We did it! Additionally, I would like to thank the esteemed members of my committee – Dr. A. Jake Lusis, Dr. Esteban Dell'Angelica, and Dr. Paul Micevych – for their assistance and direction. None of the projects here would have been possible without the many wonderful collaborators and co-authors listed below with whom I have had the pleasure of working. I have also been truly lucky to have done this work surrounded by an amazing group of scientists in the Vilain lab. Thank you to Dr. Sven Bocklandt, Dr. Alice Fleming, Dr. Saunders Ching, Dr. Francisco Sanchez, Dr. Ruth Baxter, and Dr. Valerie Arboleda. Finally, I'd like to thank the people in my life from whom I drew the strength to see this through: my family and my friends, new and old.

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Chapter 1

Introduction

Overview

Men and women are different from each other in multiple ways. These differences run the gamut from the physical (e.g. height and brain anatomy) to the psychological (e.g. cognitive abilities and behavioral traits). Sex differences that have been identified in the brain are of particular interest. While largely similar, the brains of men and women show some consistent differences. For the most part, the functional significance of the differences is unclear. However it is likely that these differences may partially explain sex differences in biochemical processes, susceptibility to neuropsychiatric diseases and disorders, and cognition and behavior. A comprehensive understanding of these sex differences, including how they originate, could provide important information to both researchers and clinicians. For example, the pathophysiology of Parkinson's disease shows marked differences between men and women. Men appear to be more susceptible to the disease – they develop it more frequently and at an earlier age than women [1-3]. There are also sex differences in the symptoms [4, 5]. Knowledge about how these sex differences emerge could give valuable insight into the process of brain development while also supplying clues about how best to manage risk factors for the disease in each sex.

In this thesis, I will explore the molecular mechanisms underlying sex differences in the brain (specifically the bed nucleus of the stria terminalis/preoptic area, BNST/POA) and behavior. In this chapter, I will provide a broad overview of the field. I will begin by discussing the paradigms and conceptual framework used to study brain and behavioral sex differences as well as describing some key findings that have led to these views. I will pay particular attention to the organizational-activational hypothesis and the classical dogma that all non-gonadal sex differences are attributable to hormonal causes. I will then

highlight some sex differences at the biological and psychological levels. Next, I will address evidence of several emerging viewpoints. The first concerns direct genetic effects, which demonstrate that factors beyond gonadal hormones can lead to non-gonadal sex differences [6]. The second viewpoint I will highlight is the compensation hypothesis as it relates to sex differences [7]. Simply put, it is the hypothesis that not all sex differences in the brain lead to sex difference in behavior and that they may be functioning to equalize behavior between the sexes instead. Finally, I will end with a discussion of the molecular mechanisms mediating the effects of gonadal hormones, focusing on the role of epigenetic mechanisms.

Sexual Differentiation

The process by which sex differences develop is named sexual differentiation. The main biological difference between the sexes is in the makeup of their sex chromosomes. Males are typically XY and females are typically XX. One could argue that all sex differences ultimately stem from this, whether the immediate factor is environmental or biological. For instance, although differential treatment and social expectations of men and women are largely responsible for sex differences in math performance, the social and the biological are intertwined [8, 9]. Men and women experience different environments because they are sorted into different social categories in a manner which largely corresponds to whether they are XX or XY.

The central dogma of sexual differentiation emerged from the work of Alfred Jost in the 1940s and it asserts that mammalian sexual differentiation is basically equivalent to sex determination, i.e. whether an individual develops testes or ovaries [10, 11]. This is one

of the earliest and most obvious manifestations of the difference in sex chromosome complement. These divergent endpoints emerge from the same primordial tissue, the bipotential gonad. Sex determination is largely genetically determined so what seems to matter most in sex determination is what genes the organism possesses [12]. In mammals, those with the testis-determining gene, *SRY*, develop testes whereas those lacking this gene develop ovaries.

Once the bipotential gonad has differentiated, the testes then produce testosterone and Mullerian inhibiting hormone (MIH). This leads to the development of male-specific internal and external genitalia (such as the penis, vas deferens, and scrotum). In the absence of testosterone and MIH, female-specific development takes place and structures such as the vagina, clitoris and oviducts are formed. This is commonly thought of as the start of the process of sex differentiation. In contrast to sex determination, sex differentiation is thought to have a strong hormonal influence and involves the development of other internal reproductive structures, the external genitalia and non-gonadal sex differences [13]. Sex-specific hormones go on to exert strong effects in many different tissues [12, 14]. Over the subsequent decades, the cumulative evidence led to the widespread belief that all non-gonadal sex differences were due solely to the actions of sex steroids. This will be referred to as the classical hypothesis and will be addressed in greater detail in a later part of this chapter. It is not surprising that the classical hypothesis retains much of its power as the changes brought about by the actions of sex steroids are profound and sometimes permanent and irreversible.

Sexual Differentiation of the Brain and Behavior

A. The Organizational-Activational Hypothesis

The dominant paradigm that has emerged with regards to hormonal effects is the organizational-activational hypothesis. It presents a unified theory of sexual differentiation of the body in mammals. As the name suggests, hormonal effects are largely divided into two: those that are permanent (organizational) and those that are acute (activational). Arguably the genesis of the idea of permanent hormone effects can be traced back to the studies of the freemartin effect in cattle by Frank Lillie [15, 16]. In opposite sex cattle twin pairs, the female twin (the freemartin) is usually infertile. The body of these freemartins differs from most females in a few ways. Their general physical appearance is intermediate between that of a male and a female . Additionally, the internal genitalia of the freemartin are masculinized although the external genitalia remain feminine. Lillie's major contribution was to discern that the masculinization of the freemartin was a case of "hormone action" [15]. Infantile gonadectomy and testicular transplant had already been shown to affect sexual differentiation by Eugen Steinach at that point but Lillie was the first to provide evidence that sexual differentiation brought about by hormones occurred prenatally. Lillie was also among the first to posit that the biological factor (testosterone) causing masculinization of the freemartins is freely diffusible through the blood, i.e. that it was a hormone. This concept was further built upon by studies in the succeeding decades from various scientists including Alfred Jost [10, 13, 17].

The organizational-activational hypothesis that is most familiar to scientists today is largely based on the pioneering work of Phoenix, Goy, Gerall, and Young [18]. We now know that in the brain, sex steroids can lead to sex differences in gene expression, neural

anatomy and morphology, and behavior [19-24]. Phoenix et al. injected pregnant guinea pigs with testosterone propionate (TP) to determine the effects of prenatal hormonal manipulation on their offspring. Studies prior to this one had used large multiple doses of hormones on the pregnant mother and her offspring [25]. In order to be able to induce the desired mating behavior of the resulting offspring and equalize hormonal conditions, the animals underwent gonadectomy. Once they reached adulthood, behavioral testing commenced. To test for lordosis, a female-typical mating behavior, the animals were injected with estradiol benzoate (EB) and progesterone to mimic the hormonal milieu required for this behavior. On the other hand, to test for mounting, a male-typical mating behavior, the animals were treated with TP. Phoenix et al. found that the prenatal TP treatment of female guinea pigs permanently displayed much lower rates of lordosis but much higher rates of mounting. These changes correspond to a defeminization and a masculinization of their mating behavior, respectively.

It is hard to overstate the importance of this study. Remarkably, the experimental framework (pre/perinatal hormone manipulation, then gonadectomy and adult hormone replacement) is still used today in many studies on sex differences in the brain and behavior with few amendments [26-28]. The conclusions that Phoenix and his colleagues came to continue to hold up to intense scrutiny and have become the de facto conceptual framework when thinking about sex differences in the brain and behavior. Firstly, this study demonstrated that masculinization of behavior (and by implication, the brain) via testosterone happens during the prenatal period. Secondly, it presented compelling evidence that these prenatal effects of testosterone are permanent as the effects of prenatal testosterone exposure were evident months after the initial hormonal manipulation.

Thirdly, it showed that there is a defined window (later termed the critical period) during which testosterone can exert these permanent effects. Postnatal or adult treatment of females cannot recapitulate these effects. These are the main concepts of the organizational theory of hormones: differentiating, permanence, and sensitivity during a small developmental period. An example of an organizational effect of testosterone is the life-long masculinization of the principal nucleus of the BNST (BNSTp) in rodents following perinatal androgen exposure [29]. In essence, testosterone (or its metabolites as we shall see later) promotes the formation of neural circuits that enable masculine phenotypes ('masculinization') and prevents the formation of those circuits that are responsible for feminine phenotypes ('feminization').

Once the relevant neural circuits have been organized, hormones can exert acute, transitory effects that are termed 'activational'. These activational effects are short-term changes that occur in the body depending on the presence or absence of specific hormones. The presence of certain hormones is able to 'activate' a particular neural circuit and drive the expression of the attendant behavior. Once the relevant hormone has been cleared from the body (or is below the threshold to activate that circuit), its effects cease. Two examples of this can be found in Phoenix et al.'s study. The first is the need for TP injections in adulthood in order to activate mounting behavior. The second is the requirement for EB and progesterone treatment of the animals to induce lordosis. We know a lot more about the activational effects of hormones than we do their organizational ones owing to the relative ease of studying them. Acute hormonal manipulations in adulthood are able to elucidate various aspects of activational effects whereas the study of organizational effects usually requires perinatal hormonal manipulation coupled with gonadectomy.

B. Expansion of the organizational-activational hypothesis

Studies following Phoenix et al.'s original findings have built upon their conclusions in several ways. The first is the expansion of the critical period and the realization that it may not be identical in all species. Phoenix et al. used guinea pigs in their study and Goy et al. later found that postnatal hormonal manipulation did not lead to the same outcomes as those taking place during the prenatal period [30]. However, in rats and mice, that window extends into the time shortly after birth (for a historical review see [25]). This was first conclusively demonstrated by Barraclough and Gorski in 1961 when they showed that neonatal injection of female rats with TP resulted in sterility due to disturbed hypothalamic function [31, 32]. This finding has been replicated and extended to other regions of the brain in many subsequent studies [29, 33-35].While there is some disagreement about the exact length of the critical period in rats and mice, it is generally accepted to close about a week after birth [36].

A second major expansion to the organizational-activational hypothesis is the idea of puberty being a second critical period (reviewed in [37]). By the 1970s, Scott et al. were positing the idea that there were multiple critical periods based on evidence from their studies on the development of social attachment in dogs and that puberty in humans was an obvious candidate for a second critical period [38]. This line of thought was furthered by Arnold and Breedlove in 1985 when they argued that hormones may also have organizing effects well into adulthood and that there are some instances where the rigid distinction between organizational and activational effects is untenable [39]. In order to establish puberty as a bona fide critical period, Schulz et al. used an experimental design similar to

the one established by Phoenix et al. except that hormonal manipulation occurred around puberty [37]. By comparing males castrated before puberty to those castrated after they were able to show that testicular hormones during puberty are important for the organization of circuits related to social and mating behavior. There is also evidence of pubertal effects on a range of behaviors in a wide variety of species including humans, rats, and mice [40-43]. Based on the accumulated evidence, Schulz et al. conclude that puberty is not a critical period separate from the perinatal one but that they are actually part of "two periods of elevated hormone secretion within a prolonged postnatal window of decreasing sensitivity to steroid hormones" [37].

A third significant addition to the initial hypothesis concerns the identity of the active molecule. The study by Phoenix et al. implicates testosterone as the hormone responsible for the masculinization and defeminization observed but the full story is more complex. Testosterone can act directly on cells through the androgen receptor. However, many of its masculinizing and defeminizing effects are actually dependent on its conversion to estradiol – commonly thought of as the 'female' hormone – via aromatization [44]. For instance, the large sex difference seen in the sexually dimorphic nucleus of the POA (SDN-POA) results from the prevention of neuronal apoptosis by aromatized testosterone [45]. Testosterone and estradiol promote sexual differentiation by acting on a wide variety of cellular processes ranging from cell division and migration to neuronal growth and survival to synaptic patterning [6].

Sex Differences in Brain and Behavior

A. Sex Differences in Neuroanatomy

The two sexes have similar but not identical brains. Most brain studies have focused on gross manifestations of these differences—namely the size of specific regions or nuclei. Yet, there is mounting evidence of sex differences at a finer level including differences in synaptic patterns [46, 47] and neuronal density [48-50]. It is beyond the scope of this chapter to provide a comprehensive review of all known neuoranatomical differences. Instead, we will review in detail the BNSTp and the SDN-POA, which are among the best-studied sexually dimorphic nuclei in the rodent brain. We have also provided other notable sex differences in the rat brain in Table 1-1. There are also excellent resources for those who are interested in delving deeper into this topic [51-53].

We have chosen to focus on neuroanatomical differences in the rat because the biological significance and origins of these differences are much clearer than in humans. Neuroanatomical differences in humans are also well-studied although ethical reasons preclude the experimental manipulations that have led to the findings detailed in Table 1-1. This significantly limits the conclusions that can be drawn from any observations made in humans.

Although these neuroanatomical differences are intriguing, most are limited because the practical or functional significance of these findings are unknown. Discovering the significance of these differences is often difficult, even in rodents. We will discuss some of these difficulties below. As science and technology continue to advance, we will eventually know how to make sense of the mounting evidence of sex differences in the

brain. For now, it is reasonable to suspect that such differences may help account for observed sex differences in behavior, neurological diseases, and cognitive abilities.

i. The Sexually Dimorphic Nucleus of the Preoptic Area (SDN-POA)

The medial preoptic nucleus, which is more popularly known as the SDN-POA, is located in the anterior hypothalamus and possesses receptors for steroid hormones [54]. It has been implicated in sex-linked processes such as male copulatory behavior [55], maternal behavior [56, 57] and gonadotropin regulation [58, 59]. The SDN-POA is the site of one of the most dramatic sex differences in the rat brain. It is a darkly staining region of the POA and is several times larger in males than in females and gonadectomized males [60]. Several lines of evidence point to this dimorphism arising as a result of differential rates of apoptosis (higher in females than males) that are regulated by estradiol [61-64]. Cell death begins in females about a week after birth and continues for about three days [65, 66].

Understanding the link between sex differences in structure and behavior is not necessarily straightforward [67]. Although the SDN-POA has been implicated in the regulation of male copulatory behavior [55], the link (if any) between the sex difference in SDN-POA size and behavior remains elusive. Masculinizing the size of the SDN-POA in female rats does not result in a corresponding masculinization and defeminization of behavior [68]. Instead, the SDN-POA may be related to inhibition of female sexual behaviors [69, 70], which might not have been an obvious hypothesis given what was known about the POA previously.

Swaab and Fliers were the first to describe a potential human SDN-POA [71]. This region was identified as interstitial nuclei of the anterior hypothalamus-1 (INAH-1) by Allen et al. but they did not replicate the findings of Swaab and Fliers [72]. Instead, they observed two other sexually dimorphic nuclei nearby: INAH-2 and INAH-3. The human equivalent of the SDN-POA is most likely INAH-3, based on replication of the sex differences in subsequent studies as well as its location, morphology and neuronal properties [73-75]. Interestingly, INAH-3 also shows a difference along the lines of sexual orientation. It is more than twice as large in heterosexual men than in homosexual ones, a difference in magnitude similar to the sex difference [74]. This nucleus is also found in sheep where it is known as the ovine sexually dimorphic nucleus (oSDN). It shows a sex difference in the same direction as in rats and humans and is organized prenatally by testosterone [76]. Furthermore, the volume of the oSDN is two times larger in female-oriented rams than in male-oriented ones [77].

ii. The Principal Nucleus of the Bed Nucleus of the Stria Terminalis (BNSTp)

The stria terminalis links the hypothalamus with the amygdala, from which it receives heavy input [78]. It is considered to be part of the limbic system. The BNST is known to be involved in the modulation of stress, gonadotropin release, and the control of male sexual behavior [79-82]. Its role in anxiety and stress behaviors is particularly well-studied [83, 84]. The BNSTp has a larger volume in male rats than in female rats [29]. As with the SDN-POA, this is due to differential apoptotic rates (higher in females, lower in males) as a result of the organizing effects of testosterone [85]. The *Bax*-dependent

sexually dimorphic cell death begins about 3 days after birth, peaking two days later and ends when the animal is a week old [86].

The BNST also shows a sex difference in humans. The central subdivision (BNSTc) is larger in men than in women, regardless of sexual orientation [87, 88]. However in male-to-female transsexuals, this region is similar in size to that of control women [87]. In addition, the size of the BNSTc in female-to-male transsexuals is close to that of control men [88]. These data suggest that gender identity, if it has a neural basis, may be related to the BNSTc and suggest that sexual differentiation of the brain and genitals may proceed in opposite directions. However, it is difficult to demonstrate a causative relationship. Based on studies of individuals with disorders of sex development who were raised as the gender incongruent with their sex chromosomes, gender identity is more heavily influenced by socialization than chromosomal sex [89, 90]. Therefore, these neural differences may just be a reflection of that individual's gender identity as opposed to its cause.

B. Sex Differences in Neurochemistry

Males and females exhibit different patterns of transmitting, regulating, and processing biomolecules. Table 1-2 presents some of the neurochemical sex differences that have been identified. As a specific example, we focus below on the monoaminergic system, which has been implicated in several neurological diseases and mental disorders that differentially affect men and women.

Monoamines are a class of small-molecule neurotransmitters that are involved in the control of a variety of processes including reproduction and sexual behavior [91, 92], respiration [93], and stress responses [94]. Monoamines have also been implicated in

numerous mental disorders, including ones that differentially affect men and women [95, 96]. Likewise, sex differences in the monoaminergic systems in the rat are well-documented. Reisert and Pilgrim have provided a comprehensive review of arguments for the genetic bases of these differences [97].

Monoamines are subdivided into two groups—catecholamines and indolamines—based on their molecular structure. The main catecholamines are dopamine (DA), norepinephrine (NE) and epinephrine, which are synthesized from the amino acid tyrosine. Figure 1-1 highlights some of the known sex differences of the dopaminergic system. Regulation of dopamine can potentially control the levels of the other two catecholamines as they are derived from dopamine.

Catecholamines are released by the adrenal glands usually in response to stress, which affects males and females differently. For instance, chronic physical stress impairs memory in male rats only [98]. The sexes also show differing neurochemical responses: Dopamine activity is upregulated in males only whereas norepinephrine is upregulated in females only (Figure 1-1A). Sex differences have also been found in the regulation and modification of dopamine (see Figures 1-1B and 1-1C). Specifically, the enzyme tyrosine hydroxylase (TH), which is involved in dopamine synthesis [99], is regulated by *Sry*—the male sex determination gene—which is not present in females. Additionally, levels of norepinehrine in the amygdala differ between the sexes as a result of age. Thus, it is likely that brain catecholaminergic responses to stress might also differ between the sexes.

Another monoamine is serotonin, which is an indolamine. Unlike catecholamines, serotonin is derived from the amino acid tryptophan. The serotonergic system shows sex differences (Figure 1-2), though many of these differences remain unlinked to behavioral

differences between men and women. Nevertheless, differences in this system likely have consequences given the link between serotonin and numerous mental disorders [100, 101].

C. Sex Differences in Cognition, Behavior and Neurological Diseases and Disorders

The consequences of the sex differences described above manifest themselves in many cognitive measures, behavioral traits, and neurological diseases and disorders. Below I will describe in detail an example of a behavioral trait and a disease that show large sex difference and use them as a way to discuss the challenges facing researchers studying these phenotypes in humans.

Aggression is often cited as an example of a behavioral trait with robust sex differences. The popular stereotype is that men are more aggressive than women and on the surface this appears to be true. For instance, men commit more violent crimes than women and that despite varying overall crime rates over time, the sex difference remains [102]. Numerous studies have also found that men are more likely to use foul language, imitate aggressive models, and engage in violence and physical aggression [103]. Sex differences in physical aggression appear to emerge at an early age, implying a biological origin for these differences [104]. However, the situation is more complex than it would first appear. The reports of sex differences in physical aggression in children are based on observational studies which may be subject to bias and prior expectations. Furthermore, even at a young age, children are subject to socialization and its attendant gendered expectations. There is also emerging evidence that men and women engage in different forms of aggression. Men seem to be more likely to respond to provocation with physical violence while women prefer to respond with indirect aggression, which involves attacking

the reputation of their targets [105]. Taken together the data suggests that the details are important when we discuss sex differences in aggression, particularly, and in cognition/behavior, generally.

A different set of difficulties face researchers studying sex differences in neurological diseases. Although environmental factors may account for some sex differences in disease, the evidence that biology plays an important role is clearer. Alzheimer's disease (AD) is the most common form of dementia and is more prevalent in women among aged individuals (refs 143, 144 from review). Additionally, there are sex differences in the clinical manifestation of AD, a complicating factor that is not uncommon among neurological diseases that show sex differences in prevalence [106-108]. Therefore, the task facing researchers is often two-fold: to understand why there is a sex difference in incidence (which can be uniform regardless of age or change with it) and to elucidate the mechanisms that lead to differences in clinical symptoms. Another issue concerns data on these diseases that have been derived from animal models. Many studies on animal models of disease have been performed using just one sex (the vast majority examine just the male of the species) leading to an incomplete picture of the biological mechanisms behind these diseases [109, 110]. This has serious consequences in the development of treatments – of the 10 drugs withdrawn between 1997-2000 over safety concerns, 8 posed higher risks to women and the higher risk in at least 4 of these drugs was likely due to physiological differences between the sexes [110]. This focus on male animals is largely due to the perception that female animals are more difficult to study due to hormonal variations that occur as part of the estrous cycle. However, it is becoming clear that researchers can no

longer afford to ignore one sex if they are interested in getting the complete picture about the disease in question [111].

Although there are undoubtedly sex differences in humans that arise due to social influences, the significance of biological factors is increasingly well-understood. Ultimately, what is most critical is a fuller understanding of the main determinants that affect the expression of sex differences which will aid our knowledge about the relationship between the brain, behavior, and environment as well as the interplay between them.

Emerging Views

A. Direct Genetic Effects

From the time of Lillie through to the last decade, the classical hypothesis, which states that all non-gonadal sex differences arise from the action of gonadal hormones, has held sway and remained the dominant paradigm. Examples of the view are rife in the literature. For instance, Jost wrote in 1970 that "[t]he developmental analysis of the body sex characteristics reveals a hormonal control [10]." As recently as 2004, Morris et al. stated that "a single factor—the steroid hormone testosterone—accounts for most, and perhaps all, of the known sex differences [112]…" Even in their ground-breaking paper from 2010 showing that the 'sex identity' of somatic cells in birds is determined by the sex chromosome of those cells and not the gonadal hormone environment, Zhao et al. repeated the claim that "the sexual phenotype of individuals is dependent on the gonad [113]…"

However, there is increasing evidence that gonadal hormones are not the sole contributor to male- and female-typical development. There is little doubt that they are the major player in this area but recent discoveries point to a role for sex chromosomes and

sex-specific genes in sexual differentiation beyond the initial step of sex determination [114, 115]. We call these direct genetic effects as they arise from the expression of X and Y genes within non-gonadal cells that result in sex differences in the functions of those cells or target cells. In addition to genes on the sex chromosomes, instances of sex-specific imprinting on autosome may also be a contributing factor [116]. Such direct genetic actions are wide-ranging and can include effects of locally produced hormones or other non-hormonal messenger molecules. For example, sex differences arising in the brain from differential paracrine secretion of neurosteroids would be considered a direct genetic effect. The commonality among these actions is that they are not dependent on mediation by hormones secreted by the gonads. In many cases, the identity of the messenger molecules have yet to be identified. I will now focus on examples in which sex differences in brain and behavior are unlikely to be influenced by only the action of gonadal hormonal secretions and may in fact be due to direct genetic effects. I will discuss evidence from studies in zebra finch and other avian species, the role of *Sry* (the male determining gene) in the brain, the Four Core Genotypes mouse model, and evidence from sex chromosome aneuploidies in humans (particularly Turner and Klinefelter Syndromes).

In Chapter 2 of this dissertation, I present data from a study we have performed in a novel mouse model of Klinefelter Syndrome named the Sex Chromosome Trisomy model that provides further support for the hypothesis of direct genetic effects on brain and behavior.

i. Evidence From the Zebra Finch and Other Avian Species

The first hint that the classical view of sexual differentiation might be incomplete came from studies of zebra finches, a species of songbird. As in many songbirds, it is only the male that sings as part of the courtship ritual. This male-only behavior has been linked to several regions of the brain that are larger in comparison to females [117, 118]. Guided by what was known at the time about sexual differentiation of behavior, researchers set out to alter the courtship behavior of these birds via the by-now classic method of hormonal manipulation. Since estrogen is needed to masculinize the song circuit, females in these experiments were treated with estrogens and/or androgens. However, these attempts were ultimately unsuccessful in causing complete masculinization [119] [120]. Only supraphysiological doses of estrogen was able to do this [121]. Castration of male zebra finches did not lead to significant differences in song development relative to intact males [122]. In fact, even the development of fully functioning testes in females did not masculinize the neural song circuitry and masculine song behavior was absent from these females [123, 124]. Further doubt about the role of hormones arose when inhibition of estrogenic action via aromatase blockers was unable to completely stop differentiation in the masculine direction [117, 125-128]

An indication that direct genetic effects played a role in the sexual differentiation of the neural song circuit came from the discovery and study of a bilateral gynandromorphic zebra finch [129]. Gynandromorphs are organisms possessing both male and female characteristics. They arise spontaneously in nature and instances have been noted in birds, insects and arachnids. One half of the bilateral gynandromorphic zebra finch's body was genetically male (ZZ) and had male-typical phenotypes in terms of its plumage, gonad and

song circuitry. The other half of its body was genetically (ZW) and phenotypically female. Therefore each side contained all the sex-specific genes that are required for the development of the associated sex-specific phenotype. Since both sides of the brain were exposed to a common hormonal environment, it is highly likely that the genetic differences in the brain cells on each side contributed to the lateral/sex differences seen in the gynandromorph. However, gonadal hormone effects were still present as both sides of the song system were larger than a normal female's.

Recent work on gynandromorphic chickens strengthens the case that the classical view largely does not apply to sexual differentiation in birds. Zhao et al. showed that the 'sex identity' (or the expression of sex-specific phenotypes) of somatic cells in birds is determined by the sex chromosome complement of those cells and not the gonadal hormonal environment [113, 129]. In mammals, transplantation of somatic cells from one sex into the gonad of the other sex reverses the sex identity of the donor somatic cells. For example, XX cells can develop into functioning Sertoli cells while XY cells can become functioning granulosa cells [130, 131]. However, this is not the case in the chicken as male donor cells introduced into the developing ovary continued to express a male-specific marker and were excluded from 'functional' structures of the host gonad. The host and donor somatic cells were exposed to the same hormones, but they responded differently based on their respective sex chromosome complement.

ii. Direct Role of Sry *in Brain Sex Differences*

In rodents, sex differences in dopaminergic neurons have been found prior to exposure to gonadal steroid hormones. During *in utero* development, rat embryos are

exposed to a plasma surge of hormones around embryonic day 17 or 18 (E17 or E18). Yet, as early as E14, dissociated cell cultures of dopaminergic neurons obtained from male and female rat brainstems were found to be fundamentally different in their morphology and function prior to exposure to gonadal steroid hormones [97]. Furthermore, females had higher numbers of dopaminergic, tyrosine hydroxylase-immunoreactive (TH-ir) cells in the midbrain; and their mesencepahlic and diencepahlic neurons produced more dopamine when compared to males. On the other hand, soma measurements of diencephalic neurons from male cultures contained larger dopaminergic neurons. These differences are not altered even when gonadal hormone levels are manipulated. A study using mesencephalic cultures from the NMRI strain of mice had similar findings [132].

Sry appears to be strongly involved in the regulation multiple components of the catecholamine biosynthesis pathway including tyrosine hydroxylase (TH), which is one of the best examples of a direct genetic regulator of a trait that differs between the sexes [133-135]. *Sry* a Y-linked gene that directs the bipotential mammalian gonad to develop as testes—hence, its name: Sex determining region on Y. Most studies on *Sry* expression have focused on the gonad and *Sry*'s subsequent effects on sex determination and differentiation [136]. Until recently, it was thought that *Sry* had no role other than sex determination. However, *Sry* expression has been found in numerous tissues outside of the testis [137-139]. This extra-gonadal expression in the adult male rat is now known to have biologically significant effects.

In a 2004 study, Milsted et al. found that *Sry* is a regulator of TH gene transcription [133]. They also demonstrated that *Sry* and TH mRNA were co-localized in the locus coeruleus, substantia nigra, and ventral tegmental area of the male rat (Figure 1-3A). By *in*

situ hybridization, our laboratory determined the spatial distribution of *Sry* mRNA within the rodent brain [134]. Specific labeling of *Sry* was observed in the substantia nigra, medial mammillary bodies of the hypothalamus, and the cortex of male rats only. These transcripts were translated and co-localized with the TH protein—all neurons in the substania nigra positive for *Sry* were also positive for TH. Knocking down *Sry* expression in the male rat substania nigra led to significantly lower TH expression and introduced a significant asymmetry in limb use where the animals strongly favored the usage of their ipsilateral limbs (Figures 1-3B and 1-3C). There was also a 26% decrease in TH-immunoreactive cells in the striatum when *Sry* expression was knocked down in that region.

iii. The 'Four Core Genotypes' Model

One of the greatest challenges in distinguishing between the organizational effects of gonadal hormones and the effects of sex chromosome complement is that the two parameters almost always co-vary. It is extremely rare to find naturally occurring cases where these parameters are decoupled. XX males who have an *SRY* translocation and XY females who have a deletion of *SRY* are some of the few examples. An animal model that has proven its utility in teasing apart these factors is the 'four core genotypes' (FCG) mouse model. FCG mice are unique because their gonadal sex is unlinked from their chromosomal sex [140]. The reason chromosomal sex and gonadal sex are able to vary independently in FCG mice is that there was a naturally occurring deletion of *Sry* from the Y chromosome found in these mice generating the Y^- chromosome. Thus the Y chromosome is no longer sex determining. An *Sry* transgene was reinserted onto an autosome. Therefore, animals

with the transgene (regardless of whether they are XX or XY), will develop as males (those that possess testes) whereas those lacking the transgene will develop as females (those that possess ovaries).

As such, in addition to XY males and XX females, there are mice in this model that are XY females and XX males. Because of this, the FCG model enables researchers to perform 2x2 comparisons where the factors are gonadal sex (presence or absence of *Sry* and hence testes) and chromosomal sex (XX or XY) and determine the relative contribution of each factor and the interaction between them. A difference between gonadal males and gonadal females can be attributed to the effects of gonadal type (which includes organizational hormone effects) and/or presence of *Sry*. On the other hand, a difference between XX and XY mice regardless of gonadal sex is attributable to chromosomal sex effects. This conceptual framework is depicted graphically in Figure 1-4.

Numerous phenotypes have been studied in FCG mice. Below, I will review one example concerning the brain and another about behavior.

Neural tube closure defects occur more frequently in females than in males [141]. As neural tube closure occurs in mice prior to gonadal differentiation, the female bias observed may be due to sex chromosome effects. In order to investigate this hypothesis, Chen and colleagues investigated the effect of sex chromosome complement on this phenotype in *Trp53* null mice on the FCG background [142]. They found that regardless of gonadal sex, XX mice had a higher incidence of lethal neural tube closure defects. Subsequently, they found that the number of X chromosomes seemed to be responsible for this difference and that the Y chromosome was not conferring a protective effect.

Recently, Bonthuis et al. studied the effect of X-chromosome dosage on male copulatory behaviors [143]. While they observed an effect of sex chromosome complement and X-chromosome number on these behaviors, their findings were rather counter intuitive. They found that XX males were displayed more ejaculations and achieved ejaculation faster than their XY counterparts. Interestingly, XX females thrust and mounted more often than their XY females. When they tested these behaviors in the Y* mouse model, which allows determination of X chromosome dosage effects, they discovered that the number of X chromosomes was positively correlated with increased mount and thrust frequencies in both sexes (for detailed information about the Y* model please refer to [142, 144, 145]). This led them to speculate that the expression levels of as yet unknown genes on the X chromosome could be causing the differences between the genotypes.

iv. Sex Chromosome Aneuploidies in Humans

Because experimental and genetic manipulation in humans cannot be carried out for ethical reasons, it is often difficult to separate the effects of gonadal sex from those of chromosomal sex. However, various sex chromosome aneuploidies exist in humans – and in a manner similar to the FCG model – these sex chromosome disorders have proven invaluable in advancing our understanding of sex differences and direct genetic effects by provide information on the role of sex chromosomes in the phenotypic differences between 46, XY men and 46, XX women.

In women, the most commonly occurring aneuploidy is Turner Syndrome (45, X). Compared to 46, XX girls, those with Turner Syndrome are more likely to have social difficulties [146]. These difficulties may be related to impairments in facial- and emotional

processing [147]. In an interesting parallel, 46, XY boys perform worse than 46, XX girls on tests of social cognitive skills [148]. Since both 46, XY boys and 45, X girls are less skilled socially than 46, XX girls, this suggests a role for X chromosome dosage on social cognition. Studies from Skuse et al. suggest that a parent of origin or imprinting effect may also be at play: 45, X girls whose X is paternally derived are more socially competent than 45, X girls whose X is maternal in origin [149]. In 46, XY boys, the X chromosome is always from the mother and could contribute to decreased social competence.

The most common sex chromosome aneuploidy in men is Klinefelter Syndrome (KS; 47, XXY), which is characterized by the presence of an additional Y chromosome. KS has been associated with a wide range of physiological and physiological abnormalities [150]. Here I will focus on findings regarding the social difficulties that KS men report having and studies of these men that suggest a role for the X chromosome in this phenotype [151-153]. Altogether the data suggest that KS men have deficits in processing socio-emotional cues, are less able to identify and then articulate their emotions but experience increased levels of emotional arousal when compared to non-KS men [152]. They are also more prone to experiencing distress during social interactions and exhibit traits associated with the autism spectrum[153]. Given the parallels between KS men and Turner women in terms of social behavioral phenotypes, it strengthens the case that the X chromosome may be involved in social cognition. However, an important caveat in the interpretation of data regarding KS men is that they have significantly lower levels of testosterone during and after puberty, which may have a great impact on cognitive development since puberty appears to be a second critical period as discussed earlier.

B. Compensation

Thus far, this review has mostly focused on sex differences in neural and behavioral endpoints. The hypothesis that underlies many of the examples put forth is that sex differences in the brain eventually lead to sex differences in behavior. However, there is an important complement to this view that we have yet to consider and that is that sex differences in the brain may also function to prevent differences in behavior between the sexes. A variation on this hypothesis is that the neural endpoint may not show overt sex differences but that the developmental process may in fact be sexually differentiated. This viewpoint was first advanced by De Vries and Boyle but has since been tested and advocated by other researchers and is termed the dual-function hypothesis of sexual differentiation [7, 154].

One example where overt sex differences in neuroanatomy do not seem to lead to a corresponding sex difference in behavior concerns the African bush shrike (*Laniarius funebris*). As discussed earlier, sex differences in the neural song circuitry in the zebra finch (larger nuclei in males than in females) appear to be linked to a sex difference in singing behavior (males sing, females do not). A similar sex difference in the size of song control nuclei is present in the shrike but both males and females sing in this species [155]. Furthermore, the complexity and repertoire of songs are similar in both sexes. Therefore, the link between the size of song control nuclei and song production is not universal and may not be as straightforward in non-zebra finch species.

The first example I will present of a sexually differentiated process leading to similarities between the sexes is X inactivation. Female mammals have two X chromosomes whereas males only have one. In order to equalize the dosage of X-linked genes between

the two sexes, one of the female's X chromosomes undergoes epigenetic modifications and becomes mostly inactive [156]. Thus, gene expression occurs largely from only one of the female's X chromosomes, compensating for the single X copy in males. My second example concerns parental care in the prairie vole. In this species, both parents take care of their pups and show no qualitative differences in parental care with the exception of nursing [157]. However, different mechanisms lead to similar behaviors in the two sexes. The hormonal changes during pregnancy are what cause female prairie voles to become responsive to pups [158]. Since male prairie voles are obviously unable to go through the same experience, they appear to have evolved a different mechanism to bring about parental care behavior. This behavior is dependent on the activation of arginine vasopressin (AVP) receptors in the lateral septum, a neuroanatomical parameter where there is sexual dimorphism – males have a dense AVP fiber network whereas females have few of these fibers [159, 160].

The recently uncovered role of *Sry* in the regulation of catecholamine biosynthesis (see above) also raises the question of compensation. We have shown that the attenuation of *Sry* expression in males results in detrimental motor effects and that females have lower levels of TH neurons [134]. However, female rats do not go through life exhibiting motor dysfunction. Therefore, a largely unanswered question concerns the identity of a female-specific 'compensatory' factor for *Sry*. The higher susceptibility of men to Parkinson's disease also implies that this factor exists and might have protective effects against the nigrostriatal degeneration that is the hallmark of Parkinson's [161]. Estrogens are a viable candidate for this factor – short-term injections of estradiol benzoate lead to an increase in TH mRNA [162] and ovariectomy results in loss of TH-positive neurons [163].

The dual-function hypothesis forces us to consider the study of sex differences in the brain a different light. Rather than just trying to associate sexually differentiated neuroanatomy and/or neurochemistry with sex differences in behavior and cognition, researchers might benefit from a careful consideration of neural regions associated with a particular behavior and studying whether those regions show any sex differences. This hypothesis also makes us reexamine the interpretation of already discovered sex differences in the brain. As described in an earlier section of this chapter, the significance of the sex difference in volume of the SDN-POA in rats is still unclear. Thus far, most studies have attempted to link it to male copulatory behavior but have met with mixed success. Instead, it may be compensating sex differences elsewhere in the brain and equalizing behavioral output.

C. Molecular mechanisms underlying the organizational effect of gonadal hormones

i. Cell death

One of the most well-studied strategies by which gonadal hormones lead to sex differences in the brain is via programmed cell death. Unlike in nematodes, lineage does not determine which cell will die but it instead appears to be a stochastic process where it is impossible to predict the survival of the cells on an individual level [164]. Sexually differentiated rates of apoptosis due to testosterone and its metabolites is responsible for the sexual dimorphism seen in the rodent BNSTp, SDN-POA, anteroventral periventricular nucleus of the hypothalamus (AVPV), and spinal nucleus of the bulbocavernosus (SNB) [165, 166]. In these regions of the brain, it is the balance between the levels of Bax (pro-apoptotic) and Bcl-2 (pro-survival) that are largely responsible for regulating cell survival

and death (exceptions for certain neuronal types exist; see [164] for more details) [45]. Bax protein levels are higher than Bcl-2 in the SDN-POA of females. As expected, this ratio is reversed in males (Bcl-2 is more abundant than Bax). Treatment with estrogen masculinizes this ratio in females and prevents subsequent developmental neuronal death. Accordingly, targeted deletion of the *Bax* gene increases the number of cells in the SNB, AVPV and BNSTp and eliminates the sex difference in cell number in these regions [164].

ii. Cellular mechanisms mediating the actions of gonadal hormones

A fascinating recent finding concerns the downstream mechanisms mediating estradiol's actions. In the POA, estradiol can act in an organizational manner to increase dendritic spine density [167, 168]. Therefore, males have higher dendritic spine density than females. The molecule responsible for mediating this effect appears to be the proinflammatory lipid molecule, prostaglandin E_2 (PGE_2). Interestingly, this mediation by PGE_2 appears to be specific to the POA. Treating females with PGE2 neonatally is also sufficient to masculinize adult copulatory behavior. The manner in which PGE2 then relays the signal to affect morphological change is shared by many other brain regions and systems [169]. This indicates that while the organizational effect of gonadal hormones act is rather unique, the molecular mechanisms by which it induces change may not be.

The discovery of PGE_2's role in sexual differentiation has sparked renewed interest in the crosstalk between the immune, nervous and endocrine systems. Long considered to be an immune-privileged organ and thus divorced from the immune system in many ways, it is now starting to become clear that the brain interacts with the immune system on multiple levels. In the POA and median eminence, prostaglandins eminence act

downstream of estradiol to facilitate the lutenizing hormone surge in adult females that ultimately results in ovulation (Ojeda et al., 1979; Rage et al., 1997). One of the sources of these prostaglandins is the microglia, the brain's resident macrophages (Adachi et al., 2009). Microglia are responsive to estrogens and are also involved in the apoptotic signaling pathways that result in the sex difference in POA cell survival [169]. Finally, disruptions in the immune system have been linked to several sex-biased neuropsychiatric traits and disorders such as addiction, autism, schizophrenia, and depression [170-173].

iii. Epigenetic mechanisms

The question of how gonadal hormones relay their messages and program cell survival, differentiation, and development has only begun to be addressed. As detailed above, the immune system and prostaglandins appear to be an important part of this process. However, given the long-lasting and irreversible nature of the organizational effects of gonadal hormones (at least in terms of morphological changes), a hypothesis that has been gaining increasing attention is that epigenetic changes are a way for these early-life events to have life-long consequences. "Epigenetics" literally means "over genetics" and refers to functional modifications to the genome that lead to changes in gene expression without a corresponding change to the underlying genetic sequence. The importance of epigenetic mechanisms has already been well-established in the pathogenesis of cancer and regulating cellular differentiation in other contexts [174, 175]. Increasingly, it is seen as the mechanism by which the environment can effect rapid, heritable, transgenerational changes which has important implications for evolutionary theory [176]. Epigenetic mechanisms can take several forms, the most well-known of which are histone

modifications, microRNAs (miRNAs), and DNA methylation. All three have been implicated in sexual differentiation of the brain and I will review the evidence for each in this section of the chapter.

Histone modifications were one of the first types of epigenetic mechanisms to be directly implicated in the process of brain sexual differentiation. Histones can be covalently modified at many different residues and in several different ways including phosphorylation, methylation, sumoylation, ubiquitylation, and acetylation. Although our knowledge is increasing at a rapid rate, the interactions between these different modifications is complex and remains poorly understood. One of the leading hypotheses is that the different modifications have a combinatorial effect and form a "histone code" that is then "read" by the cell and translated into biological outputs [177]. What is clear is that histone modifications are critical in establishing and maintaining the spatial architecture of DNA within the nucleus, which has enormous effects on gene expression [178].

Histone acetylation has emerged as one of the leading candidates for histone modifications that are critical to brain sexual differentiation. Histone acetylation/deacetylation is one of the major epigenetic regulators of gene expression. The addition of acetyl groups to the lysine residues on histone tails is catalayzed by histone acetyltransferases (HATs) and is strongly associated with active transcription [179]. These acetyl groups are removed by histone deacetylases (HDACs) which leads to repression of gene expression. In 2009, Tsai et al. showed that males had higher levels of histone acetylation in the developing cortex/hippocampus than females and that females treated with testosterone in utero were masculinized [26]. Murray and colleagues then demonstrated that sexual differentiation of the BNSTp was dependent on regulation of

histone acetylation and that disruption of this process also led to alterations in olfactory behavior in female mice [27, 180]. Importantly, although the disruption of histone acetylation/deacetylation was generalized throughout the brain, there were significant changes only in sexually dimorphic vasopressin cell groups [180]. Finally, HDAC inhibition results in a reduction in intromission in male rats [181]. Altogether, these data suggest that histone acetylation plays an important role in sexual differentiation of the BNSTp, vasopressin cell groups and male copulatory behavior.

A second epigenetic mechanism that has been shown to play a crucial role in sexual differentiation of the brain is miRNAs which act as post-transcriptional regulators. Each miRNA can have more than a hundred different mRNA targets. miRNA genes are generally transcribed by RNA Polymerase II, generating a stemloop containing the primary miRNA [182]. This early form of the miRNA can range from several hundred nucleotides all the way to tens of kilobases. The primary miRNA is then processed by the Microprocessor complex to produce the hairpin precursor miRNA, which is cleaved and processed via the Dicer complex to produce the ~22nt mature miRNA. Finally, the mature miRNA is loaded into RISC to guide the complex to its target mRNAs. If there is at least near-perfect complimentarity between the miRNA and the target mRNA, then the target is degraded. miRNAs are already known to be involved in mammalian sexual development [183]. *miR-29b* is enriched in the ovary and negatively regulates *Dnmt3a* and *Dnmt3b*, the *de novo* methyltransferases, during the period of XY-specific *de novo* methylation [184]. In the brain, *miR-124* regulates adult neurogenesis in the subventricular zone by targeting *Sox9*, which is the immediate downstream target of *Sry* in sex determination [185].

As miRNAs ultimately stem from genes, they can also be regulated by gonadal hormones. When estrogen receptor alpha (ERα) binds to the upstream regulatory regions of miR-21 and miR-23a, they are expressed in breast cancer cells [186]. Morgan and Bale identified 149 miRNAs with sex-biased expression in the neonatal mouse brain [187, 188]. Of these 47 appeared to be regulated by sex chromosome complement. Interestingly, early prenatal stress causes a transgenerational dysmaculinization of the expression of several miRNAs in the brain and an associated dysmasculinization of gene expression. miRNAs also regulate cellular processes that are important for brain sexual differentiation, such as *COX-2* expression, which is induced by estradiol and is required for establishing sex differences in dendritic spine density in the POA [188]. In conclusion, the ability of miRNAs to regulate a large number of genes, their responsiveness to gonadal hormones and sex chromosome complement, and apparent involvement in masculinization of the brain make them extremely promising candidates to gain further insights into sexual differentiation of the brain.

Finally, I will turn my attention to what we know about the role of DNA methylation in brain sexual differentiation. DNA methylation is the addition of a methyl group to the five-carbon of a cytosine, usually in the context of a CpG dinucleotide. When this happens in the promoter region of a gene, DNA methylation is predominantly associated with transcriptional repression. However, methylation in gene bodies appears to be associated with active expression [189]. DNA methylation is established and maintainted by a family of enzymes known as DNA methyltransferases. In mammals, DNMT1 is thought to be the family member that is responsible for maintenance of methylation. DNMT3a and DNMT3b are considered the main *de novo* methyltransferases [190]. Once methylation has occurred

at promoter sites, methyl-binding proteins, such as MeCP2, and co-repressors are recruited to form repressive complexes [191]. DNA methylation is critical in the regulation of gene expression. For instance, it is the molecular mechanism underlying imprinting, or parent-of-origin, effects in which there is preferential expression from one parental allele over the other [192]. There are sex-specific parent-of-origin effects on allelic expression in the brain although the contribution (if any) of gonadal hormones and/or sex chromosome complement have yet to be determined [116]. Aberrant DNA methylation is strongly associated with many diseases, including cancer and might even contribute to its pathogenesis [175, 193].

Of the various epigenetic mechanisms we know of, DNA methylation has arguably the longest history and strongest association with sexual differentiation. X inactivation, one of the most marked sex differences, is heavily reliant on DNA methylation to maintain repression of the inactive X chromosome [194]. In fact, the initial evidence concerning the effect of gene body methylation came from studies of X chromosome inactivation [195]. Recent studies are beginning to implicate DNA methylation in brain sexual differentiation. The DNA methyl-binding protein, MeCP2, is expressed at higher levels in female rats compared to male rats in the amygdala and the ventromedial hypothalamus postnatal day 1 [196]. This sex difference disappears by day 10 of life. Since the sex difference is present only during the perinatal critical period, it suggests MeCP2 may be involved in brain sexual differentiation. Studies taking a more direct look at the relationship between DNA methylation and brain sexual differentiation have mainly used candidate gene approaches and focused on the promoters of ERα and progesterone receptor (PR), partially due to technical limitations. ERα expression in the medial POA of female offspring is affected by the

amount of maternal licking/grooming (LG) [197]. Those who receive high levels of LG have greater levels of ERα expression than those who receive low levels of LG. This difference emerges in infancy and persists into adulthood. There is a correlated difference in *ERα* promoter methylation – lower levels of methylation were seen in female offspring who received high LG.

Thus far, only one study has directly tested if the organizing effect of gonadal hormones is evident in changes in DNA methylation patterns. Schwarz et al. used the conceptual framework first laid down by Phoenix et al. to test the effect of neonatal estradiol exposure on DNA methylation at the promoters of *ERα*, *ERβ*, and progesterone receptor (*PR*) in the POA and mediobasal hypothalamus in female rate [198]. What they found was that two CpG sites in the promoter of *ERα* showed a sex difference during the critical period and that estradiol treatment of females led to a masculinization of methylation at these sites. In adulthood, a different site showed sex differences and appeared to be affected by neonatal estradiol exposure. At the *ERβ* promoter, none of the three CpG sites surveyed displayed clear signs of hormonal modulation. However, in adult animals, methylation at one of the CpG sites was higher in males than in both females and females who had been treated with estradiol, suggesting a sex chromosome effect at this site. Finally, they saw no sex differences in PR promoter methylation.

It is becoming clear that the interactions between gonadal hormones and epigenetic mechanisms is complex and widespread. Therefore, what is currently lacking is an unbiased, genome-wide survey of the effect of early hormonal exposure on DNA methylation. In Chapter 3 of this dissertation, I will present a study we have performed that addresses this point.

Conclusion

The significance and importance of the role of gonadal hormones in sexual differentiation of the brain and behavior is undeniable. The organizational-activational hypothesis put forth by Phenox and colleagues in 1959 has withstood decades of rigorous testing and continues to be the dominant paradigm in this field. Relative to the rich scientific body of work about gonadal hormones, the importance and contribution of chromosomal effects have only just begun to be understood. However, it is becoming increasingly clear that gonadal hormones are not the only significant contributing factor to brain sexual differentiation and that a full understanding requires a consideration of sex chromosome effects. Additionally, the molecular mechanisms that underlie both of these factors also remains poorly understood.

This dissertation seeks to elucidate those molecular mechanisms. Data will be presented in Chapter 2 to demonstrate the effect of an additional X chromosome on partner preference behavior and gene expression in the adult brain in the Sex Chromosome Trisomy model, a novel mouse model of Klinefelter Syndrome. We show that partner preference is feminized in these mice. We also show that a significant proportion of genes are feminized in their expression pattern. We then investigate the effect of the interactions between the additional X chromosome and the Y on gene expression. Together these data can help identify candidate genes for Klinefelter-related phenotypes and also elucidate how sex chromosome complement affects gene expression in the brain and subsequent behaviors. We then turn our attention to the effect of gonadal hormones on the genome. We demonstrate that there are sex differences in DNA methylation patterns throughout the genome. We then show that neonatal exposure to testosterone can affect methylation

status of a large number of genes and that some sex differences in DNA methylation are due to non-hormonal factors. Finally, we saw a profound effect of developmental stage and that this factor can interact with hormones to affect methylation patterns.

FIGURES AND TABLES

Figure 1-1: The catecholaminergic pathway is sexually differentiated. TH: Tyrosine hydroxylase, L-DOPA: L-dihydroxyphenylalanine, NE: norepinephrine. (A) Chronic physical stress results in sexually dimorphic responses. Dopamine (DA) activity is upregulated exclusively in males (light blue arrow) while norepinephrine activity is upregulated exclusively in females (yellow arrow) [98]. (B) Control of TH expression differs between the sexes. SRY, the testis determining gene, which is not found in females, directly regulates TH expression [133, 134] while 17β-estradiol increases TH expression only in males (light blue arrows) [199]. Aromatase activity is more responsive to dihydrotestosterone (DHT) in males than in females (dark blue arrow) [200]. (C) Male rats have higher NE levels than female ones in the amygdala (A) and hypothalamus (HT) early in life [201]. As the rats age, the direction of this difference is reversed.

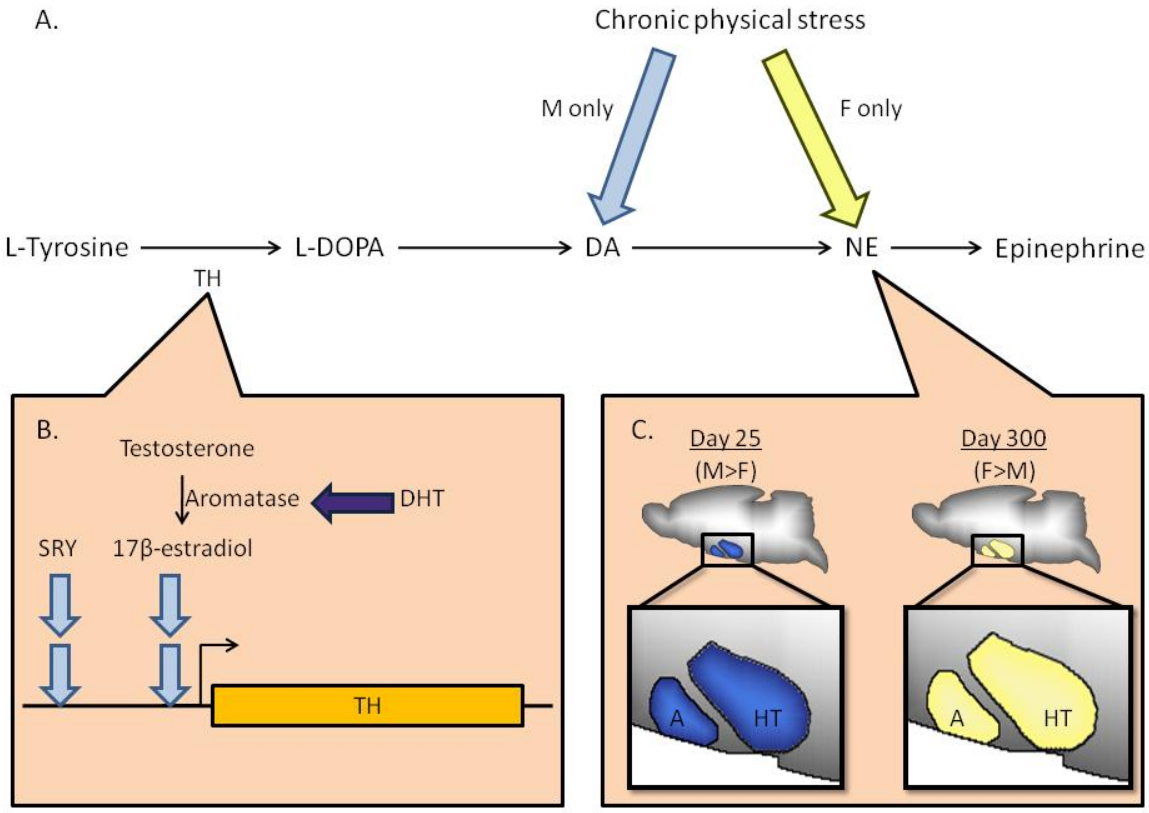
A.
Chronic physical stress
M only
F only
L-Tyrosine
L-DOPA
DA
NE
Epinephrine
TH
B.
Testosterone
Aromatase
DHT
SRY
17β-estradiol
TH
C.
Day 25
(M>F)
Day 300
(F>M)
A
HT
A
HT

Figure 1-2: Serotonin (5-HT) is sexually differentiated on multiple levels. In addition to the differences illustrated above, some of the loci that influence 5-HT levels in the blood are also sexually dimorphic [202]. References: 1 - [203], 2 - [204], 3 - [205], 4 - [206].

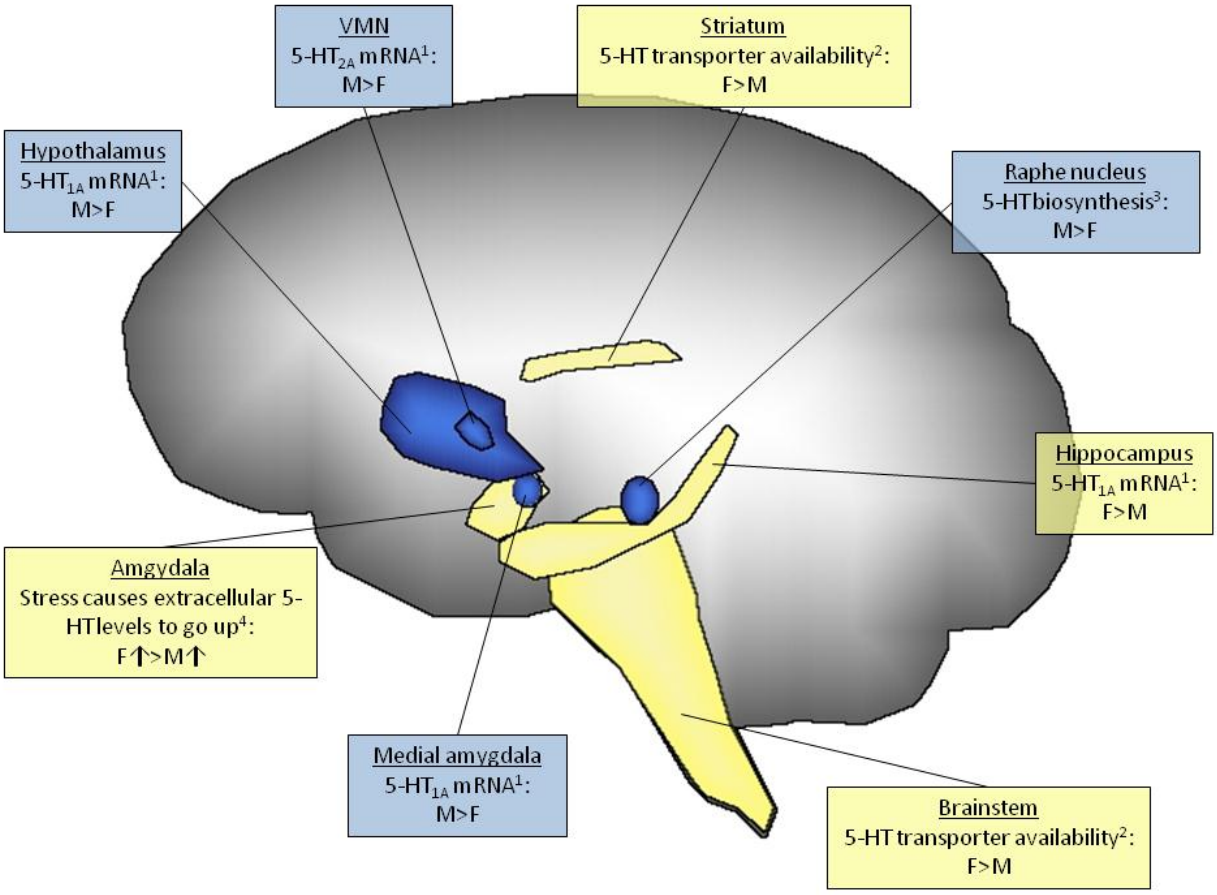

Figure 1-3: *Sry* regulates tyrosine hydroxylase (TH) levels and motor behavior. (A) Sry and TH colocalize in the locus coeruleus (LC), ventral tegmental area (VTA) and substantia nigra pars compacta (SNc). (B) Knockdown of Sry expression in the SNc leads to a reduction in the number of TH-immunoreactive (TH-ir) neurons. Unilateral infusion of antisense oligodeoxynucleotides (ODN) against *Sry* decreased the number of TH-ir neurons by 38% compared to the contralateral side infused with sense ODN. (C) Unilateral downregulation of TH expression by Sry leads to asymmetric limb use. Animals preferentially used the forelimb ipsilateral to the side of the antisense ODN infusion (preferred limb highlighted in yellow) .

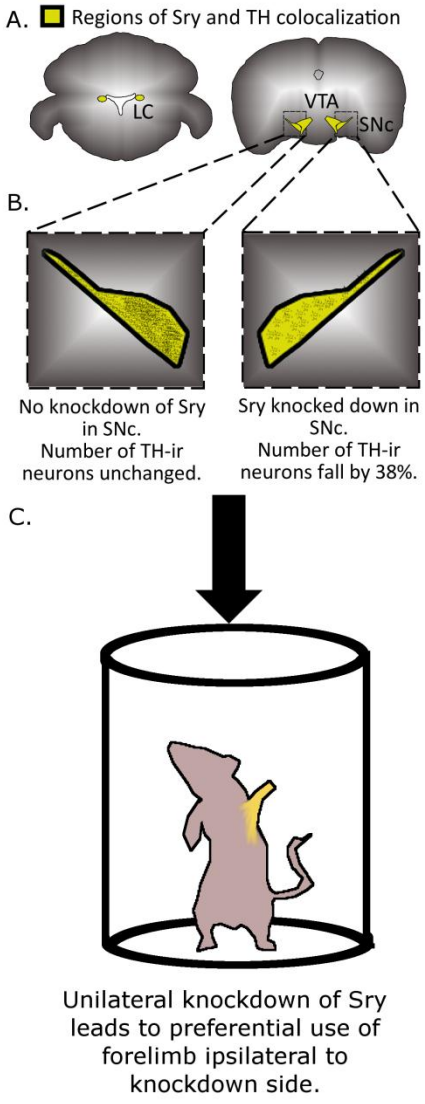

Figure 1-4: The four core genotypes model. This model enables a 2x2 comparison where the independent factors are gonadal sex and sex chromosome complement.

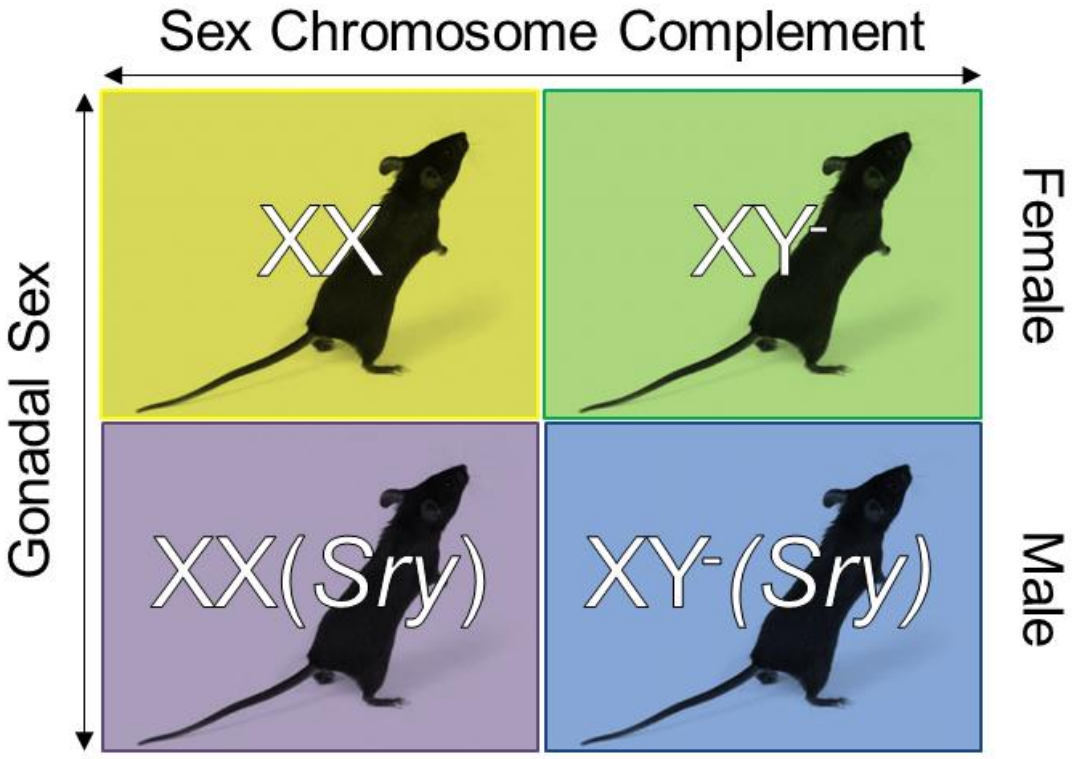

Table 1-1: Selected neuroanatomical sex differences in the rat.

Structure/Region	Known roles	Sex difference	Basis of difference
Sexually dimorphic nucleus of the Preoptic Area (SDN-POA)	The POA is implicated in the regulation of male copulatory behavior [112]. Lesions of the SDN alone slow acquisition of this behavior. Potential human equivalent is INAH-3 [72].	2.6 times larger in males [60].	Perinatal aromatized androgen decreases neuronal apoptotic rates in males [45].
Anteroventral Periventricular Nucleus (AVPV)	Involved in regulating the luteinizing hormone surge in females [45] and male copulatory behavior [207].	2.2 times larger in females with a higher cell density [208].	Degeneration of cells in this region is greater in males [165] due to prenatal action of androgen
Bed Nucleus of Stria Terminalis (BNST)	Plays a role in the control of male sexual behavior [80], release of gonadotropin [79], and modulation of stress [81, 82].	The principal nucleus (BNSTp) is larger in volume in males [29].	The larger volume in males is due to sexually different apoptotic rates caused by testosterone [85].
Corpus Callosum	Conducts information between the two halves of the cortex [209].	Larger in neonatal males [210].	Organizational effects of testosterone lead to masculinization while feminization appears to be dependent on estrogens [211, 212].
Arcuate Nucleus (ARC)	Helps regulate the estrus cycle [213], appetite and body weight [214].	Neurokin-B neurons innervate capillary vessels in the ventromedial ARC in post-pubertal males only [47].	Dihydrotestosterone is responsible for the masculine projection pattern [215].
Amygdala	Strongly associated with emotion, decision-making and Pavlovian conditioning [216].	Adult males have a larger medial nucleus than adult females [217].	Treatment of females with estradiol masculinizes this nucleus [217].
		The posterodorsal aspect of the medial amydala is 65% larger in males [34].	Activational effects of circulating androgens accounts for the larger region in males [218].
Cerebral cortex	Connected to a wide range of processes from memory [219] to language [220] to emotional processing [221].	Right posterior cortex is thicker than left but only in males [222].	Gonadal hormones play a role in maintaining the sex difference (ovariectomy masculinizes the cortex of females) [222].
Ventromedial Hypothalamic Nucleus	Involved in the control of lordosis, mounting, and norepinephrine release [223].	Females have less synapses in the ventrolateral VMN	Organizational effects of aromatized testosterone appear to be crucial in

(VMN)	High concentrations of steroid receptor mRNA have been observed in the ventrolateral VMN [224].	compared to males [49].	establishing the masculine trait [225].
Substantia nigra pars compacta	Made up almost entirely of dopaminergic neurons. Dopamine is involved in control of motor activity [226].	Females have 20% fewer dopaminergic neurons [134].	A genetic component has been demonstrated in mice [227].

*Note: This table highlights some prominent sex differences in the rat brain but it is by no means exhaustive. Conflicting evidence concerning the examples reported here (particularly in the SDN-POA) exist, and the interpretation of the data is often more complicated than this summary implies.

Table 1-2: Selected neurochemical sex differences in the brain.

Neurochemical system/pathway	Known roles	Species	Selected sex differences
Cathecolamines (also see Figure 1-1)	Involved in the control of a variety of processes including reproduction and sexual behavior [91, 92], respiration [93], and stress responses [94].	Rat	Male have higher norepinephrine (NE) levels in the amygdala and hypothalamus at day 25. Direction of this sex difference is reversed at day 300 [201].
			In response to chronic physical stress, dopamine (DA) activity is upregulated only in males whereas NE activity is increased only in females [98].
		Human	Women appear to be more dependent than men on NE for long-term emotional memory formation [228].
Serotonin	Modulates a wide variety of processes including mood, aggression, perception, reward, and attention [229].	Rat and human	Sex differences in the serotonergic system are found at multiple levels [202-206]. See Figure 1-2 for an illustration of some of these differences.
Aromatase	Plays a key role in sexual differentiation of the brain by converting testosterone to 17β-estradiol[44].	Rat	Aromatase activity is higher in males than females in many regions including the anterior hypothalamus, BNST and POA [230].
			Only males experience spikes in the expression of brain-specific and total aromatase during embryonic development and shortly after [231].
Vasopressin (VP)	VP in the central nervous system (CNS) has been linked to learning, memory and motor behavior [232]. It has also been connected to the control of social behaviors such as pair-bonding, parenting and aggression [233].	Rat	The number of vasopressin-positive cells is 2 to 3 times higher in males than in females [234].
			Vasopressin-positive projections are also 2 to 3 times denser in males [234].
			Intrahypothalamic release of VP due to an increase of plasma osmolality is higher in females. [235]
		Human	Some studies have found that plasma VP concentrations are higher in men than in

			women [232].
Cholinergic system	The cholinergic system helps regulate the sleep-wake cycle and modulates synaptic plasticity implicated in memory, learning, and development [236, 237]. Sex differences are found at many points in the cholinergic system [reviewed in 232].	Rat	Levels of acetylcholine (ACh) are higher in females, regardless of estrous cycle, than in males [238]. The maximal level of Ach in females was found at proestrus.
			The binding affinity of muscarinic Ach receptors is lower in females than in males [239]. Estrogens appear to modulate the binding activity of these receptors [240].
		Human	Men are more sensitive to cholinergic stimulation than women [241].
Opioid system	Opioids are a class of chemical for which receptors are found throughout the CNS [242, 243]. Opioids exert an analgesic effect and also play a role in stress response and reproduction [244].	Rat and mouse	Generally, μ and κ class opioids seem more effective in males than females although in some cases the effectiveness is equal [245]. In a minority of cases, they are more effective in females.
		Human	μ-opioids appear more effective in women than in men [245].
			μ-opioids show significantly higher binding potential in women in the amygdala, thalamus and the cerebellum [246]. The sex difference in the first two regions is reversed after menopause.

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Chapter 2

Partner preference and brain gene expression in XXY males from a novel mouse model of Klinefelter Syndrome is feminized

Like most modern scientific projects, this work was collaborative, with important contributions from my colleague, Negar Ghahramani, and members of Arthur Arnold's lab at UCLA. However, I was the lead in the intellectual and performance aspects of the behavioral tests. In terms of the data/figures related to that portion of the project, I was responsible for the design and preparation of Figures 2-1 and 2-2, and Tables 2-2 and 2-3.

Introduction

Klinefelter Syndrome (KS) is characterized by the presence of an extra X chromosome in men resulting in a karyotype of 47, XXY. It has a frequency of 1:426 to 1:1000, making it is the second most frequent chromosomal aneuploidy in live births after trisomy 21 and the most frequent sex chromosome aneuploidy in humans [1-5]. The source of the additional X is usually non-disjunction during parental gametogenesis (~97% of cases) with paternal and maternal non-disjunction contributing equally to instances of KS [1]. The remaining occurrence of KS arise from errors in mitotic division in the zygote [1]. KS men experience hypogonadism and are almost always infertile. Follicle-stimulating hormone and lutenizing hormone levels are elevated but testosterone (T) levels are significantly lower, starting at puberty [1]. KS men usually present with eunuchoidal proportions, small testes and penis, sparse to absent body and facial hair, and feminine distribution of body fat (including gynecomastia) [1]. However, since the onset of androgen deficiency can differ between individuals, there is often some variability in clinical presentation.

Cognitive, psychosocial, and neurological traits among KS men are also not uniform [6]. Common deficits include (1) language difficulties, which are present in 70-80% of KS patients and thus are the most common problem encountered ; (2) lower verbal IQ than

performance IQ (i.e. visuospatial skills tend to be better than verbal skills); (3) impaired executive functions ; (4) more sensitivity, anxiousness and insecurity, and vulnerability to depressive disorders than general population men [6-9]. In terms of psychosocial functioning, KS boys appear to be more at risk for behavioral difficulties than controls and tend to do worse on the Social Communication Questionnaire, which is a validated first-level screen for autism spectrum disorders (ASD) [10]. Overzealous attention to detail, impairment in the ability to decode facial expressions and interpret affective tone of voice have also been reported [11-13]. This combination of phenotypes closely resembles traits in individuals with ASD and in line with this, KS boys are more likely to be diagnosed with ASD [6].

Several traits in KS men more closely resemble the female-typical pattern than the male one. In this dissertation, we will refer to those traits as being feminized. Firstly, KS men tend to have a body fat distribution more akin to women and many experience gynecomastia [1]. Secondly, the risk of systemic lupus erythematosus (SLE) in KS men is ~14-fold higher than in 46,XY men, which is similar to the risk in 46,XX women [14]. Although KS has not yet been shown to be significantly associated with other autoimmune diseases, which are typically strongly female-biased, numerous lines of evidence indicate being XXY may be a risk factor [15-18]. Thirdly, there is an increased rate of homosexual behavior and gender non-conformity among KS men [19-21]. Ratcliffe et al. and Bancroft et al. observed that KS boys have more problems relating to same sex peers, less expression/identification with typically masculine traits, and lower sexual interest in girls compared to controls [9, 19]. Similarly, Schiavi et al. found that KS men had a more negative attitude towards conventional gender roles during childhood and were judged by

others to be less masculine and manifesting less self-acceptance in adulthood with respect to controls [21]. Finally, more KS than controls report engaging in sexual acts with other men [20, 21].

A greater understanding of the factors that lead to the phenotypes associated with KS are crucial for better clinical management. This information could also benefit our knowledge concerning sex differences in neurological disease, cognition and behavior. Differences between KS and general population men can be ultimately traced to the presence of the extra X chromosome, the lower levels of androgens during puberty or the interaction of these two factors. However, at present, the pathophysiology of KS is still poorly understood and the interventional experiments required to differentiate between the possible causal factors of KS features cannot be carried out in humans for obvious ethical reasons. Additionally, investigating the consequent genetic and biochemical changes in relevant tissue and at the correct time points is difficult – at best – in human patients. Therefore, the use of an animal model is essential. In this dissertation, we describe the results of the first experiments performed on a novel mouse model of KS called the Sex Chromosome Trisomy model. In SCT mice, gonadal sex is decoupled from sex chromosome complement, which is one of main advantages this model. In brief, we are able to generate animals with the following genotypes: XX, XY, XXY, and XYY, all of which can be gonadally male (with testes) or female (with ovaries) (see Figure 2-1A and Methods section for more details). Hereafter, we will designate the gonadal sex of the animal using either an M or and F. For example XXY males will be designated XXYM whereas the females will be XXYF.

Although there are already several existing mouse models of Klinefelter Syndrome (namely the Y* model [22, 23] and the XXY model developed by Ronald Swerdloff's group

[24-26]), we believe ours has several key advantages. In Swerdloff's XXY model, generation of the relevant mice involves a difficult four-generation breeding scheme. In contrast, the SCT model has the potential to generate all 8 possible genotypes from a single mating pair. The other important advantage of the SCT model is that it enables separation of the effects of gonadal status/hormones from sex chromosome effects. This allows discrimination of the effects of sex chromosome trisomy that interact with gonadal sex from those that are independent. We are also able to determine the effect of sex chromosome number (2 vs. 3), effect of Y dosage (0, 1, or 2), effect of X chromosome dosage (1 vs. 2), and determine if there are any interactions between these different factors on the phenotype in question.

In this study, we investigated whether XXY males from the SCT model are more feminized than XY males on a behavioral phenotype and a molecular one. The behavior we chose to investigate was partner preference. Sexual orientation or partner choice (the terms will be used interchangeably in this dissertation) is one of the most sexually dimorphic behaviors in the Animal Kingdom. Almost all males choose females as sexual partners and vice versa [27]. Furthermore, little is known about the molecular basis of partner choice. The evidence that exists strongly implicates the X chromosome as playing a role in male sexual orientation [28, 29]. Sexual orientation is also feminized in KS men. Therefore, it is a strong candidate to test for behavioral feminization in XXYM. Thus, the SCT model has the potential to elucidate the biological underpinnings of partner choice, especially those related to the X chromosome. We hypothesize that the increased rate of homosexual behavior in KS men will manifest itself in this mouse model as an altered preference for estrus females over males in XXYM. This could take the form of a lower interest in or attraction to estrus females compared to XYM, increased interest in or

attraction to males, or a combination of both changes. XYY animals were excluded as there is no evidence of either increased homosexual behavior or altered gender role behavior in XYY men. Analysis of the male groups revealed that XXYM spent significantly less time with the stimulus female than XYM. There was also a trend for XXYM to spend more time with the stimulus male compared to XYM.

We next investigated gene expression in XYM, XXM, XXYM and XXF. We studied the combined bed nucleus of the stria terminalis and preoptic area (hereafter BNST/POA) a region in the brain known to be involved in a number of sexually dimorphic traits and responsive to the actions of both gonadal hormones and sex chromosome complement. The BNST/POA includes two highly sexually dimorphic nuclei that are responsive to the permanent, organizational effects of testosterone and estradiol (its aromatized form) and which are sexually dimorphic in a wide range of species [30-33].

We found that gene expression in the BNST/POA of XXYM is not generally feminized. However, a small but highly significant proportion of genes that show a basal sex difference are feminized in XXYM. In addition to these feminized genes, we also found that many other genes were differentially expressed between XXYM and XYM and that the majority of these differences can be attributed to the interactions between the additional X chromosome and the Y chromosome.

Materials and methods

Animals.

All experimental procedures using mice were approved by the UCLA Chancellor's Animal Research Committee. All mice used in this study are from the MF1 outbred strain

and were bred in the Life Science Vivarium at UCLA. The SCT model is only viable on an outbred strain. The initial MF1 stocks were a gift from Dr Paul Burgoyne, MRC National Institute for Medical Research, London. The mice were kept at a 12:12 light: dark cycle with food and water available ad libitum. In the SCT model, gonadal sex (whether the animal has testes or ovaries) is unlinked from the presence of the Y chromosome in these mice. This is because the testis-determining gene, *Sry*, has been deleted from the Y chromosome, resulting in a Y^- chromosome [34]. In some mice, *Sry* is present as a transgene inserted into an autosome. Therefore, animals that lack this transgene (even if they have the Y^- chromosome) develop ovaries and are defined as females. Those with the *Sry* transgene (even if they lack the Y^- chromosome) develop testes and are classified as males [35].

The initial cross that led to the generation of this model was between an XX female and an XY^-Y^* male on an MF1 background (for details of the Y* model, please refer to [36-38]). One of the genotypes generated from this initial cross were XXY^- females, which were fertile. We then crossed XXY^- females to an XY^-*Sry* male. The XXY^- females produced two types of eggs: X and XY^-(as the second X chromosome always segregates with the Y^-). On the other hand, the XY^- *Sry* males produced four types of sperm: X, X*Sry*, Y-, and Y^- *Sry*. Therefore, offspring had one of four sex chromosome complements (XX, XY^-, XXY^-, or XY^-Y^-) and approximately half the offspring inherited the *Sry* transgene and developed as male while the other half will developed as female.

As with the Four Core Genotypes mouse model, these mice enable a comparison of animals that have the same sex chromosome complement but different gonadal types [39]. Additionally, this model allows comparisons that elucidate the effect of sex chromosome number (two vs. three), the number of X chromosomes (one vs. two), and the number of Y

chromosomes (none vs. one vs. two) on any trait of interest. In this study, we have focused on only XX males and females, XY^- males, and XXY^- males.

Karyotyping

The genotype of offspring was determined by karyotyping of cells from cultures of ear clippings. A small piece of tissue from the ear was removed using sterile scissors and then digested in collagenase. The samples were then transferred to 60mm tissue culture dishes and culture medium (DMEM with 10% FBS, 0.5% pen/strep, and 1% fungizone) was added to each sample. The cultures were incubated at 37°C and 5% CO2 until the cells reached 80% confluence (about 4 days). Colcemid was added to halt the cells in metaphase. After synchronization, cells were trypsinized, harvested and fixed with in an ice-cold 3:1 methanol-glacial acetic acid mixture at -20°C overnight. To make metaphase spreads, cell suspensions were dropped onto clean glass slides from a height of 3-4 inches. The slides were aged for 2-3 days, stained with Giemsa and evaluated using light microscopy at a magnification of 400x. The chromosome count and number of Y chromosomes was then determined. The Y's were discernible because they are smaller and darker than the other chromosomes. 40 chromosomes with no Y's was designated XX, 40 with one Y was XY, 41 with 1 Y was XXY, 41 with 2 Y's was XYY.

Surgery, hormone replacement and tissue dissection

All mice used in this study were gonadectomized bilaterally between 97 and 124 days of age. At the time of gonadectomy, a silastic capsule (1.57 mm inner diameter × 2.41 mm outer diameter) filled to 5mm in length with crystalline testosterone (T) was implanted into the neck of each mouse. Following surgery, each animal was housed individually.

Tissue collection was performed 4 weeks after gonadectomy (between 125 and 152 days of age). Mice were anesthetized with isoflurane and then immediately decapitated. Whole brain was rapidly removed from the skull and brain regions of interest were dissected under a microscope, ventral side down on an ice-cold slide. After removal of the dura mater, two cuts through the brain along the coronal plane were made. The first was at the midpoint of the optic chiasm (0.14 mm anterior to bregma) and the second was where the optic tract enters the brain (0.58 mm posterior to bregma). The resulting slab of tissue was then placed posterior side down. The BNST/POA was defined as the region ventral to the lateral ventricle and bounded laterally by the medial edge of the internal capsule. After dissection, the tissue was immediately placed on dry ice and stored at -80C until it was processed for downstream experiments.

Testosterone assay

Samples were collected at the time of euthanasia. In all cases, blood was obtained from the carotid artery following decapitation. Blood samples were then processed to isolate serum and stored at -20C until assays for testosterone were performed. Testosterone assays using radioimmunoassay were performed by Ligand Assay and Analysis Core at the University of Virginia Center for Research in Reproduction (supported by NICHD (SCCPIR) Grant U54-HD28934). Testosterone measurements were performed in singlet reactions using Siemens Medical Solutions Diagnostics testosterone RIA with a reportable range of 0.72-111.00 ng/L. There were no significant differences in measured testosterone levels between our experimental groups using one-way ANOVA ($F(5, 70) = 1.53$, $p=0.1955$).

Partner preference testing

8-13 animals from each genotype were underwent partner preference testing. All animals were sexually naïve. For a schematic of the testing apparatus see Figure 2-1B. All preference tests were conducted in a Plexiglas box measuring 8 x 8 x 36 in. At each lateral end of the apparatus, a partition was inserted to create a chamber measuring 4 x 8 in. Each end chamber housed a single stimulus animal. These end chambers were separated from the main chamber by removable clear Plexiglas dividers with evenly-spaced ½-in. air-holes. Contact between the stimulus and test animals was thus prevented but auditory, visual and olfactory stimulation could be communicated. The area 0-5 in. away from the stimulus animal's chamber was defined as the "incentive zone", which was marked by a length of tape [40].

Stimulus males were left gonadally intact and were sexually experienced. All stimulus females were ovariectomized and injected subcutaneously 48 and 24 hr before testing with estradiol benzoate (1.25mg/kg mouse; dissolved in sesame oil) followed by progesterone (1mg per mouse; dissolved in sesame oil) 3 hr before testing began to induce estrus. All behavioral tests were performed between 9:00 A.M. and 1:00 P.M., which was right after the end of the dark cycle. In order to avoid possible end bias, the sex of the stimulus animal at each end was randomized between test animals.

The testing took place one to two weeks following gonadectomy and testosterone replacement. At the beginning of each test, the test animal was placed in the testing apparatus in the absence of stimulus animals for 10 min to adapt to the testing apparatus and to ensure that there is no development of any end preferences. After this period of acclimatization, the stimulus animals were placed into the side chambers, one sex on each

side. Experimenters then left the room and the test animal's behavior was recorded for 5 minutes using a digital camera.

The test animal was allowed to roam the testing apparatus freely and choose between the two stimulus animals or spend its time in the large middle compartment away from the stimuli. We quantified the time that the test animal spent within each incentive zone and used this as our measure of time spent with that stimulus animal. The amount of time that all four limbs of the test animal were within the incentive zone was recorded. In addition, the number of times the test animal crossed the incentive zone marker towards the stimulus animal on that side was counted. The observer was blinded to the identity of the test animals. A random selection of tests were rescored by an independent observer. Observations of time spent never varied by more than ±2 seconds. Number of crossings into the incentive zone determined by the two observers matched exactly in all instances.

The position of the stimulus animals were varied between tests. After each round of testing, the apparatus was cleaned thoroughly with 70% ethanol. The apparatus was also cleaned with water at the end of each day.

Non-parametric tests were used as the data were not normally distributed. Data from male and female test animals were analyzed separately. In both sexes, XXY animals were first compared to XY ones and the between group comparisons were then expanded to include XX mice. Relative preference for one stimulus sex within a group and comparisons of time spent with each stimulus animal between XXY and XY were determined using the Mann-Whitney U test. For comparisons with a strong expectation of directionality (e.g. XYM had been shown by prior work to prefer a stimulus female over a

male), we used a unidirectional test. When the between group comparisons was expanded to include the XX groups, we used the Kruskal–Wallis one-way analysis of variance test.

Microarray data processing

8 samples from each genotype were analyzed. All samples that were included had undergone the partner preference testing described earlier in this manuscript. Gene expression was surveyed using the MouseRef-8 v2.0 Expression BeadChip Kit (Illumina, San Diego, CA, USA, catalog no. BD-202-0202). Total RNA was isolated from mouse brain tissue using Qiagen AllPrep DNA/RNA Mini Kit (catalog no. 80204). Quantity and quality of isolated RNA was determined by Agilent 2100 Bioanalyzer RNA Pico assay. cDNA synthesis, labeling and hybridization steps were performed by the UCLA Neuroscience Genomics Core. Microarray data were processed using R. Data was first background corrected using the *nec* function in the *limma* package [41]. Probes with low-quality data (detection p-value of >0.05 in more than a third of the samples) were then removed from the dataset. Next, batch effects were corrected using the ComBat R script and the data underwent quantile normalization and $\log_2$ transformation using the *limma* package [42].

Determination of feminized genes in XXYM

Genes that passed a cutoff ≥1.2 fold and p≤0.05 between XYM and XXF were deemed to be sexually dimorphic in their expression [43, 44]. The p-value was determined using the two-tailed Student's t-test. The mean expression value of each gene that met the criteria was rescaled so that it equaled 0 in XYM and 100 in XXF. We converted the expression values using the following formula:

$$y=Ax+B$$

where y=the rescaled expression value (0=XYM, 100=XXM), x=normalized $\log_2$ expression value, A=constant 1, B=constant 2. Once A and B were determined the mean expression value for that gene in XXYM was calculated using the formula. This resulted in a feminization score for each sexually dimorphic gene in XXYM. Feminization scores closer to 0 meant the gene had a more masculine expression pattern while a score closer to 100 meant a gene was more feminine. Scores were capped at -25 and 125. We chose a score of 70 as the lower threshold for considering a gene a candidate for feminization in XXYM, as that was the lowest score among the X inactivation escapees detected as sexually dimorphic in our dataset. Feminized gene candidate gene then had their feminization score evaluated using a one-sample t-test (H_0: expected feminization score is 0) and corrected for multiple testing using the Benjamini-Hochberg method (FDR=10%) [45]. The heat map of expression patterns of feminized genes was generated in R using the heatmap.2 function from the gplots package[46]. Script available on request.

Determination of genes affected by being XXY

Two pairwise comparisons (XXYM vs. XYM and XXM vs. XYM) were performed and genes that passed a cutoff ≥1.2 fold and p≤0.05 (by the two-tailed Student's t-test) in each comparison was determined. We used GeneVenn (http://genevenn.sourceforge.net/ , [47]) to determine the dissimilarities and overlap between the two comparisons. The data were then analyzed using Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com).

Quantitative reverse transcription–polymerase chain reaction (qRT-PCR)

500 nanogram of total RNA was used as a template to perform reverse transcription using the Tetro cDNA Synthesis Kit (Bioline, Taunton, MA, USA, catalog no. BIO-65043)

according to the manufacturer's instructions. The RNA samples used for validation were from the original microarray samples contingent on availability (n=6-9 per genotype). The primer sequences used are detailed in Table 2-1. All primers used spanned at least one intron. Glyceraldehyde-3-phophate dehydrogenase (GAPDH) was used for normalization of gene expression between samples. qRT-PCRs were carried out in duplicate utilizing the Syber Green-based SensiMix SYBR No-Rox Kit (Bioline, catalog no. QT650-05) according to the manufacturer's instructions. For all reactions, the cycling conditions were as follows: initial denaturation and activation at 95C for 10 min, and then 40 cycles of denaturation at 95C for 15 sec, annealing at 62C for 15 sec and extension at 72C for 15 sec. We used the standard curve method to determine relative expression and assessed significance using the Student's t-test (α=0.05). Data are expressed as fold change where the expression level in XYM has been set to 1.

Results

Partner preference in XY males

The timeline for this study is presented in Figure 2-1C. In order to test for partner preference in our mice, I used a three-chambered apparatus. In brief, a stimulus animal of each sex is placed at each end of the apparatus and the test animal is placed in the center chamber and allowed to choose between the two stimulus animals or remain in the center. I recorded the test and determined how much time the test animal spent within 5 inches of the stimulus. The results of these tests are summarized in Table 2-2.

To validate this experimental setup, we tested whether we could detect the expected preference for estrus females in XYM [24, 48]. As expected, XYM spent significantly more time with the stimulus estrus female than with the stimulus male (U=39, p=0.010 by the unidirectional Mann-Whitney; Table 2-2).

Partner preference in XXY and XX males

I first compared XXYM to XYM as this is a clinically relevant dyadic comparison. XXYM did not show a preference for the estrus female. Instead, there was a trend for XXYM to spend more time with the stimulus male than with the stimulus female (U=46, p=0.14 by the Mann-Whitney test; Table 2-2).

I then analyzed time spent with each stimulus sex separately. Based on the higher rates of homosexual behavior seen in KS men, I hypothesized that XXYM would spend less time with the stimulus female and a greater amount of time with the stimulus male when compared to XYM. I found that part of this hypothesis was borne out. XXYM spent significantly less time with the stimulus female than XYM (U=23, p=0.018 by the unidirectional Mann-Whitney test; Fig. 2-2A). Although the difference in time spent with the stimulus male was not statistically significant, there was a trend for an increase in XXYM on this measure (U=35, p=0.12 by the unidirectional Mann-Whitney test; Fig. 2-2B). Taken together, these results suggest that XXYM are less attracted to estrus females and more attracted to males when compared to XYM.

The analysis was then expanded to include XXM. This group did not show a clear preference for estrus females and spent similar amounts of time with both stimulus animals of both sexes (U=36, p= 0.71 by the Mann-Whitney test; Table 2-2). XXM were not

significantly different from the other groups in either time spent with the stimulus male or female based on the Kruskal-Wallis test.

Effect of genotype on motor behavior

Klinefelter patients show deficits in their motor skills including running speed, visual motor control, response speed and motor speed [49-51]. Therefore, a potential source of the observed differences in partner preference between XYM and XXYM may be due to deficits in motor function. In order to rule out differences in locomotor function, I measured the number of approaches the test animal made to the stimulus animals. An approach was defined as each time the test animal crossed distance markers on either side of the testing apparatus towards a particular stimulus animal (the marker was 5 inches away from the stimulus). The data are summarized in Table 2-3. No significant between-group differences were seen in the number of approaches made to the stimulus female, stimulus male or the overall total. Additionally, there were no significant within-group differences in number or approaches to the female vs. the male.

Assessment of the degree of feminization of gene expression in the BNST/POA of XXY males

We then turned my attention to gene expression phenotypes in the brain of XXYM. We started by investigating if the gene expression profile in XXYM male mice is feminized and if so, what the extent of this feminization is. We examined a region of the brain that is highly sexually dimorphic: the BNST/POA. We first determined genes that display basal sex differences. These were defined as genes with >1.2-fold difference between XYM and XXF, and $p<0.05$ by the Student's t-test [43, 44, 52]. This resulted in a list of 216 (Supp. Table 2-1).

The mean expression value of these sexually dimorphic genes was rescaled so that it equaled 0 in XYM and 100 in XXF. Then their expression in XXYM was recalculated along this scale which resulted in each gene being assigned a feminization score between -25 and 125 (see Methods for details). If that gene received a score of 70 or higher, it was deemed a candidate for feminization. We chose 70 as a cutoff because this was the lowest score of a sexually dimorphic, known X-inactivation escapee in our dataset. The distribution of feminization scores is visualized in Fig. 2-3A. The expression patterns of the majority of sexually dimorphic genes in XXYM more closely resemble XYM than XXF (153 of 216 genes score below 50) so gene expression in XXYM is not generally feminized. However a minority of genes scored 70 or above (30 in the BNST/POA). We then performed the one-sample t-test on the feminization scores followed by Benjamini-Hochberg correction (FDR=10%) on this list. Genes that survived this correction were deemed feminized. This resulted in 27 of 216 sexually dimorphic genes in the BNST/POA being categorized as feminized genes. The expression patterns of these feminized genes in XXYM, XYM, and XXF are visualized as a heat map in Fig. 2-3B. The proportion of feminized genes is much higher than would be expected ($p<0.0001$ by the chi square test for both regions). Among these feminized genes were *2610029G23Rik*, *Eif2s3x*, *Kdm6a*, *4933439C20Rik* and *Xist*. The first four are known X-inactivation escapees and *Xist* is critical for the silencing of the inactive X chromosome [53]. Taken together, these data imply that the process of X-inactivation occurs normally in XXYM in the BNST/POA.

Differential expression of a number of feminized genes was verified using qRT-PCR. Those results are consistent with the microarray data (Fig. 2-4).

Determination of the effects of being XXY on gene expression in the BNST/POA.

I was also interested in determining the effect of the additional X chromosome in XXYM on gene expression in the BNST/POA and to investigate if there were differentially expressed genes between XXYM and XYM beyond the feminized ones. Analysis of gene expression between these two genotypes revealed a large number of genes that were differentially expressed ($p<0.05$; fold>1.2). There was a total of 190 differentially expressed genes. 29 genes were more highly expressed in XXYM than in XYM, whereas 161 genes had lower expression (Supp. Table 2-2). In addition, many of the genes that were detected as differentially expressed were autosomal indicating that escape of X-inactivation may not explain all the differences observed in gene expression.

I then performed functional analysis on our dataset using Ingenuity Pathway Analysis to functionally annotate these differentially expressed genes and to characterize the pathways that were different between XXYM and XYM. 8 of the top 10 pathways affected by being XXYM were related to immune function (Table 2-4).

The differences in gene expression between XXYM and XYM may have arisen from several sources. The first and most obvious difference between these animals is the presence of the additional X chromosome in XXYM. This additional X may act on its own or in concert with other chromosomes (autosomes and/or the Y). There may also have been differences in hormonal levels during the time periods where the brain is sensitive to the organizational effects of testosterone and its metabolites. Although we did not obtain measurements of testosterone throughout the lives of our animals, it is likely that the lower testosterone levels seen in KS males are recapitulated in XXYM given that other mouse models of KS show this phenotype [26, 54].

In order to differentiate between these sources, we performed a second pairwise comparison to find genes differentially expressed between XXM and XYM. 164 genes were differentially expressed between XXM and XYM (Supp. Table 2-3). Next, I compared these lists to the ones generated by XXYM vs. XYM (Fig 2-5). We found that of the 190 genes different between XXYM and XYM in the BNST/POA, 170 were unique to that comparison (Fig 2-5, yellow). Genes that are unique to XXYM vs. XYM (i.e. those affected uniquely by being XXYM) are likely to be those affected by interactions between the additional X and the Y, and/or testosterone deficiency. Furthermore, since we only observed a significant difference in partner preference between XXYM and XYM, these uniquely affected genes may be candidates for genes associated with this partner preference phenotype and other traits unique to KS/XXYM.

On the other hand, genes detected as differentially expressed in both the XXYM vs. XYM and XXM vs. XYM comparisons (i.e. affected by being a 2X male) are likely affected directly by the presence of the second X chromosome and/or its interactions with autosomes. They are also less likely to play a large role in KS/XXYM-specific traits. 20/170 genes are affected by being a 2X male (Table 2-5 and Fig 2-5, green). The direction of the change of genes affected by being a 2X male almost always matched between the two pairwise comparisons – if a gene was upregulated in XXYM relative to XYM, it was also upregulated in XXM compared to XYM. Some of these 2X male genes are known X-inactivation escapees but most are autosomal. This demonstrates that the effects of the additional X can be genome-wide and not just confined to the sex chromosomes.

Discussion

In this study, we present a novel mouse model for the study of sex chromosome aneuploidies termed the Sex Chromosome Trisomy, or SCT, model. As some traits in KS men are feminized, we investigated the extent of feminization in male mice from the SCT model in their partner preference and gene expression in the brain.

To test for feminization of partner preference, I used a tri-compartment apparatus that prevents physical contact between the test and stimulus animals. This allowed me to examine just the approach aspect of the partner preference behavior [40]. This setup is similar to the one used in [24] but differs in two main ways. The first is that the test animal does not have to pass through a doorway to approach its chosen stimulus animal. This means that the test animal does not have to first explore a chamber to learn the sex of the stimulus animal that inhabits it. As a result, my experimental setup is more efficient as the test animal needs to use less of the testing period to learn what is on the lateral sides of the apparatus. The second is that I used live animals as opposed to used bedding to test for partner preference. Although used bedding has been utilized in a large number of studies examining partner preference, it relays olfactory cues only [24, 48, 55]. The use of live animals, as in our study, enables the communication of visual and auditory cues – in addition to olfactory cues – which are important components of mating behavior [48, 56]. The use of live animals may present some potential confounds because it may introduce social approach and interest components to a test for sexual partner preference. However, approach behaviors are an important part of partner preference and under naturally occurring conditions, social and sexual components that affect partner preference are always in play simultaneously [40]. Therefore I reasoned that the use of live animals over

used bedding best recapitulated the process by which mice choose their partners and more closely captures the varied cues that humans use in making decisions about their partners.

I first tested if partner preference was feminized in XXYM compared to XYM. I observed that XXYM did not display the preference for estrus females seen in XYM and further investigation revealed that XXYM were significantly less interested in or attracted to the stimulus female than XYM. Additionally, there was a trend for XXYM to be more interested in or attracted to the stimulus male with respect to XYM. XXM were not significantly different from either XYM or XXYM on any of our measures. This implies that the feminization of partner preference in XXYM is not due solely to the presence of the additional X chromosome but rather interactions between this chromosome and the Y. Furthermore, I observed no differences in partner preference between the female groups (data not shown). This implies that the differences observed between XXYM and XYM are reliant on perinatal androgenization. This is in stark contrast to a recent study that found an increase in X chromosome dosage was positively associated with the expression of male copulatory behaviors (mounting, thrusting, and ejaculation) independent of gonadal status/perinatal androgenization and the presence of the Y chromosome [22]. Therefore it appears that the effect of an additional X chromosome is different between partner preference (where it appears to feminize in conjunction with the Y) and copulation (where it masculinizes the behavior independent of the Y). This difference is not unexpected as approach (partner preference) and consummation (copulatory) are distinct aspects of sexual behavior and may be regulated differently.

There may have been differences in hormonal levels between XXYM and XYM starting at puberty that could have caused differences in the organization of brain regions

relevant to this behavior and led to its subsequent feminization. Although we did not measure hormonal levels in our animals prior to gonadectomy (and thus are unable to distinguish between the direct effects of sex chromosome makeup and those of differing androgen levels), there is reason to suspect hypoandrogenization in XXYM as two other mouse models of KS show reductions in androgen levels [26, 54]. The role of androgen and its metabolites in establishing partner preference in mice is demonstrated by the abolishment of a preference for odor from estrus females in male mice lacking functional aromatase [48]. In future studies, it will be of great interest to examine gonadal hormone levels throughout the lives of SCT animals. It is important to note that the behavioral differences that I observed are very unlikely to have arisen from the transient effects of circulating hormones as all test animals underwent gonadectomy and received testosterone implants. There were no differences in the levels of testosterone following surgery. However, I cannot definitively rule out that the circulating testosterone may have had differing effects based on genotype.

A recent study from Liu et al. examined sex preference in a different mouse model of KS [24]. The authors found that castrated XXY and XY male mice who received testosterone (a treatment paradigm similar to ours) preferred odors from estrus females over those from males. Thus, it seems that in order to fully manifest the feminized partner preference that we have observed the presence of olfactory, visual and auditory cues are required. Another possible source of the difference between the studies is the genetic background of the mice used (C57BL/6J in the Liu et al. study and MF1 in ours), which can cause differences in behavior [55]. Liu et al. also concluded that it is social rather than sexual traits that are affected in XXY males [24]. If that is also the case with SCT mice, it would

appear that a social preference for male mice is able to overcome the sexual cues from the receptive stimulus female when a live animal is used as a stimulus. However, it is unclear if social traits are altered in our model. Thus, we are currently investigating social recognition. If the results of those tests indicate that social recognition is not altered in XXYM, we can infer that it is sexual partner preference that is affected. On the other hand, if the data indicate that social recognition is different in XXYM compared to XYM, further testing of partner preference using non-receptive females will be informative and aid in the interpretation of our data.

In the second part of this study, we investigated gene expression in the BNST/POA of XXF, XXM, XYM, and XXYM animals. The principal nucleus of the BNST and the sexually dimorphic nucleus of the POA are both larger in males as a result of higher rates of programmed cell death in female animals in the absence of testosterone and its metabolites during the perinatal critical window [57, 58]. The POA is implicated in the regulation of male copulatory behavior whereas the BNST is involved in the control of male sexual behavior, gonadotropin release and the modulation of stress, all of which are traits that show large sex differences [27].

We wanted to examine the extent of feminization of gene expression in the brain of XXYM. First, we found that although gene expression is not generally feminized in XXYM, there is a small but significant proportion of genes with expression patterns that more closely resemble XXF than XYM in both regions of the brain. Feminized genes the BNST/POA are involved in apoptosis, regulation of cell cycle/proliferation, and neurodevelopment and function. For instance, *Spag9* (also known as *JLP*), is feminized in the BNST/POA (feminization score of 100.2) and interacts with N-cadherin and links it to

p38 MAPK signaling [59]. This interaction appears to be important in the avoidance of synaptic loss, which can lead to neuronal death and strongly correlates with decreased cognitive function. It also plays a role in neurite outgrowth in response to nerve growth factor [60]. We speculate that the feminization of gene expression in the brain may ultimately be reflected in the feminization of behaviors and other traits in XXYM/KS. The source of this feminization in expression is likely largely due to the presence of the additional X chromosome but may also be related to presumed differences in androgen levels between XXYM and XYM.

Next, we sought to elucidate biological pathways that were different between XXYM and XYM. Immune function pathways were those that were most significantly affected by the XXY genotype. These immune-related pathways were mostly involved in adaptive immunity. A minority was involved in both innate and adaptive immunity and only one played a role exclusively in innate immune response. It is intriguing that pathways which play a role in both innate and adaptive immunity feature prominently in the datasets because emerging evidence indicates that there is crosstalk between the immune, nervous and endocrine systems (reviewed in [61]). Estradiol has long been known to be the key endocrine agent in setting up sexual dimorphism in various regions of the brain including the POA, BNST, anteroventral periventricular nucleus, and ventromedial hypothalamic nucleus [27]. Only relatively recently have the molecular events downstream of estradiol begun to be elucidated. One of the most fascinating findings has been that estradiol's masculinizing effects in the POA and on sexual behavior are mediated by prostaglandin E_2 (PGE_2), a proinflammatory lipid molecule [62, 63]. Furthermore, microglia, the brain's resident macrophages, are responsive to estrogens and are involved in the apoptotic

signaling pathways that ultimately lead to the sex difference in POA cell survival [61]. Many gender-biased neuropsychiatric traits and disorders such as addiction, autism, schizophrenia, and depression have also been linked to disruptions of the immune system [64-67].

PGE2 is able to take part in both the innate and adaptive immune systems through its interactions with its receptors (EP1-4) although only EP2 and EP4 are necessary for masculinization of the POA and attendant behaviors [61, 68]. Our findings indicate that crosstalk between the nervous and immune system is not just important for brain sexual differentiation but that differences in these pathways due to the presence of the additional X chromosome may be responsible for the some of the divergence between XY and KS men. The apparent involvement of these immune pathways is not necessarily an indication that the immune systems of XXYM/KS men are dysregulated compared to XY males. Rather, we hypothesize that there is overlap in the mechanisms used in nervous system development and in immune function and that some of the present distinctions are merely an artifact of which system it was identified in first. An important followup to these findings will be manipulate these pathways *in vivo* in animal models and investigate the consequences on both behavior and brain morphology.

I also identified genes that were uniquely affected by being an XXY male (as opposed to those affected in common in XX and XXY males). This distinction allows us to pinpoint candidate genes for phenotypes associated with XXYM/KS men but not XX males. I hypothesize that the genes that are the best candidates for further exploration will be those that are both feminized and uniquely affected by being XXYM. There are four such genes: *Cdc45l*, *Hsd3b2*, *Serpinh1*, and *Thoc3*. *Hsd3b2* is of particular interest because its gene

product is important for the synthesis of several steroid hormones such as progesterone, androstenedione, and testosterone. The expression of this gene is lower in both XXF and XXYM compared to XYM. Much attention has been paid to differences in circulating androgen levels between KS and general population men. However, to my knowledge there have been no studies on whether synthesis of steroids in the brains of KS men is changed from their XY counterparts. The action of locally synthesized steroids has been likened to neurotransmitters so small changes in local synthesis could potentially have a domino-like effect and lead to dramatic differences in downstream phenotypes [69]. The observation that *Hsd3b2* is feminized only in XXYM indicates that feminization of local synthesis of androgens may be a potential novel mechanism leading to KS phenotypes.

A fascinating question that I am not able to address with the SCT model is what role, if any, are played by parent-of-origin effects. The SCT breeding scheme always results in each parent contributing one X chromosome each. In KS patients, this is not always the case: since about maternal and paternal nondisjunction account for a similar number of cases, about half of KS men have one X chromosome from each parent (as in SCT mice). The other half has two X chromosomes of maternal origin. Parent-of-origin effects may help explain some of the variability seen in KS. There is already compelling evidence that these effects have large impacts on behavioral traits in women with Turner Syndrome (45,X). Skuse et al. were the first to demonstrate these effects when they showed that Turner women with a paternally derived X (45, X^P) performed better on some verbal and higher-order executive function skills relative to those whose X was of maternal origin (45, X^M) [70]. Since then, other groups have also noted differences between 45,X^P and 45, X^M women on cognitive and physical phenotypes [71-73]. There is less information about parent-of-

origin effects in KS but Bruning et al. found differences in autistic and schizotypal traits between KS dependent on the parental origin of the additional X chromosome [74]. A further complication is non-random X inactivation which happens in at least a subset of KS patients and may amplify these effects [75]. Animal models will be of great importance to help fully elucidate the extent of the influence of parental origin.

The feminization of several physiological traits in KS men hints that there may be feminization on a molecular level as well. Our findings support this view and demonstrate that interactions between the additional X chromosome and the Y in XXY contribute to the feminization of KS behavioral and molecular phenotypes. Such information is crucial in elucidating not only the pathophysiology of KS, but also the origin of sex differences in brain and behavior.

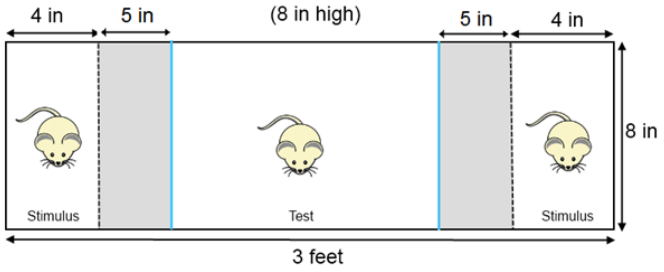
FIGURES AND TABLES

Figure 2-1: The experimental setup used for this study. A. The breeding scheme used to generate SCT mice. Mothers are XXY- and produce two types of eggs: X and XY-. Fathers are XY-Sry and make four types of sperm: X, XSry, Y-, and Y-Sry. This results in four possible sex chromosome complements in the offspring: XX, $XY^{-,}$ XXY^{-}, or $XY^{-}Y^{-}$ all of which can be with or without Sry. **B.** The three-chambered apparatus for partner preference testing. A stimulus animal (either male or an estrus female) was placed in each lateral chamber. The chambers holding the stimulus animals are separated from the large middle chamber by clear, perforated dividers. The test animal is placed in the middle chamber where it is free to choose to spend time close to either stimulus animal (in the gray incentive zones) or by itself in the middle. Time spent in each incentive zone was recorded and used as a measure of time spent with that stimulus animal. **C.** Study timeline. At about postnatal day 100, all mice used in the study underwent bilateral gonadectomy and received an implant of a silastic capsule filled with testosterone. Behavioral testing began a week after surgery. At about 128 days of age and after completion of behavioral testing, blood and tissue collection was performed.

A

		Type of egg		Sex of offspring
		X	XY^-	
Type of sperm	X	XX (**XXF**)	XXY^- (**XXYF**)	**Female**
	XSry	XXSry (**XXM**)	XXY^-Sry (**XXYM**)	**Male**
	Y^-	XY^- (**XYF**)	XY^-Y^- (**XYYF**)	**Female**
	Y^-Sry	XY^-Sry (**XYM**)	XY^-Y^-Sry (**XYYM**)	**Male**

B



C

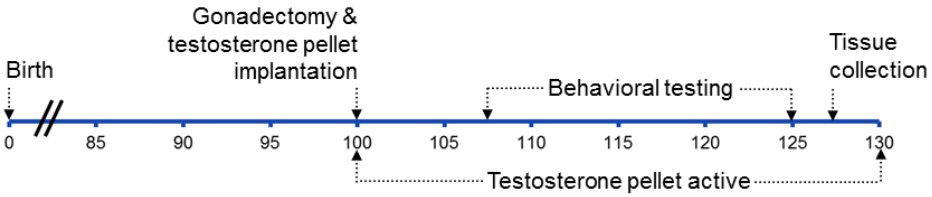


Figure 2-2: Time spent with the stimulus animal. The boxplot depicts time spent with the stimulus animal of each sex. Lines through the boxes indicate the median time in each group. Whiskers represent the limits of the upper and lower quartiles. **A.** XXY, but not XX, males spend significantly less time with the stimulus female compared to XY males. **B.** Median time spent with the stimulus male is higher in XXY compared to XY males.

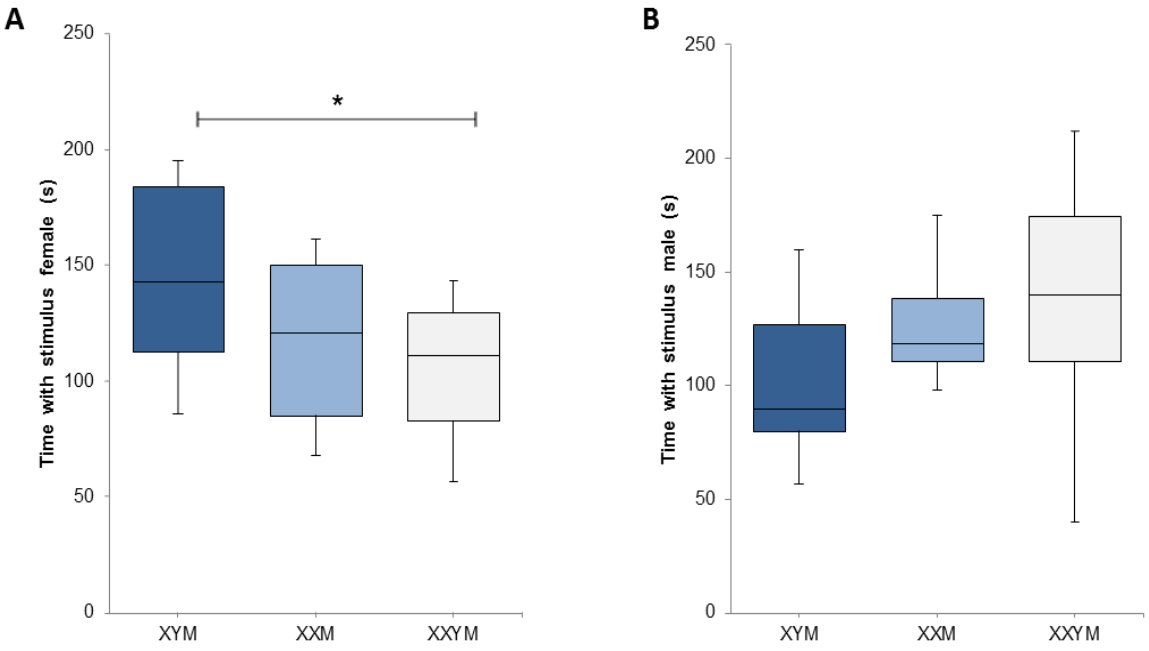


Fig. 2-3: Assessment of the degree of feminization of brain gene expression in XXYM. Mean XXYM expression of sexually dimorphic genes were assigned a feminization score and plotted on a continuum between average XYM and XXF expression in **A.** Dots represent the mean score of each gene in XXYM while the lines represent the standard error. The color of the dot indicates whether expression of that gene is closer to the male (blue) or female (red) end of the spectrum. Genes considered feminized are indicated by the dashed box. A feminization score of 70 was used as the lower boundary for categorizing a gene as feminized. The expression pattern of feminized genes that survive Benjamini-Hochberg correction (FDR=10%) are visualized using a heat map is shown in **B**.

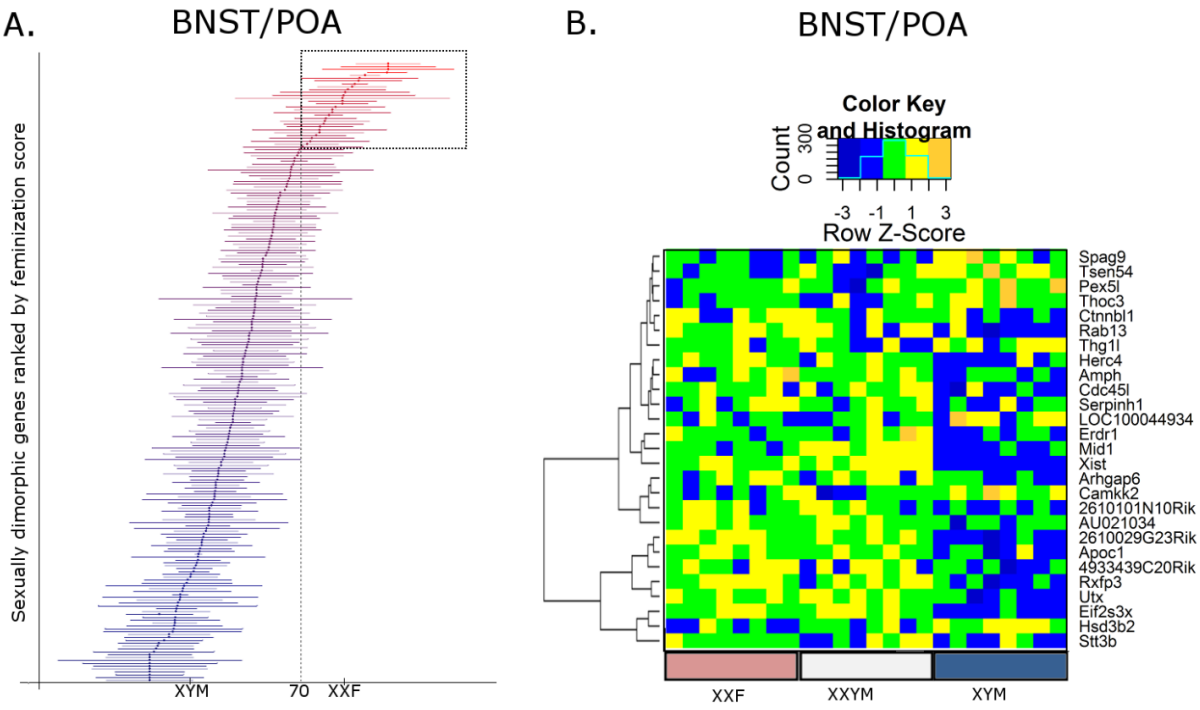


Fig. 2-4: Quantitative RT-PCR confirmation of feminized genes in XXY males. mRNA levels of Eif2s3x and Utx in XY males, XX females, and XXY males in the BNST/POA were analyzed. Error bars represent the standard error from 6-8 biological replicates from each group. Expression is relative to GAPDH and is normalized to XY males. *p<0.05 by Student's t-test.

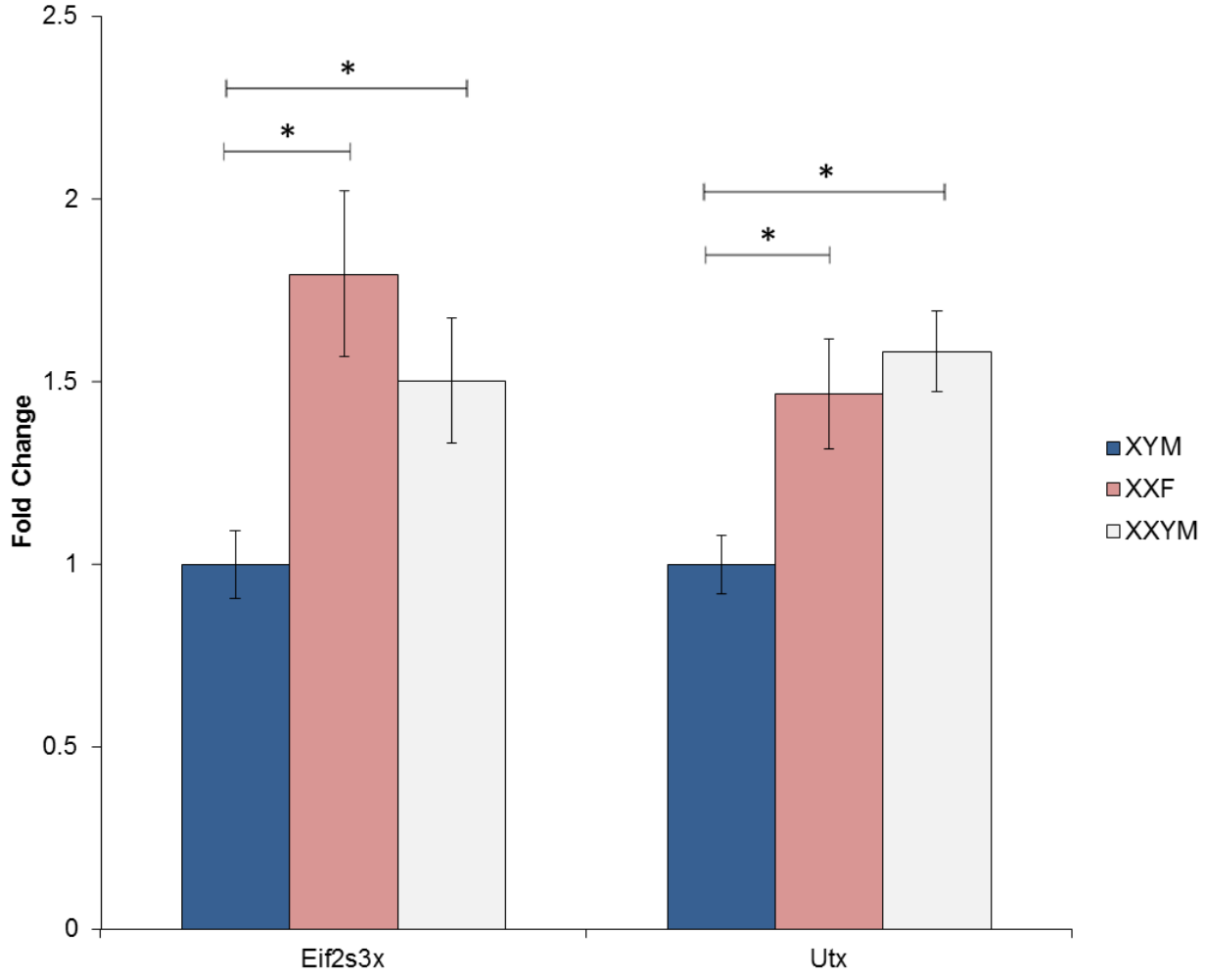


Fig 2-5: Determination of genes affected uniquely by being XXY. The Venn diagram shows genes that are differentially expressed (>1.2-fold, p<0.05 by Student's t-test) between XXY and XY males (yellow), and between XX and XY males (blue) in the BNST/POA. This identified 20 genes that are different between XXY and XY due to direct effects of the additional X chromosome and/or its interactions with autosomes (green).

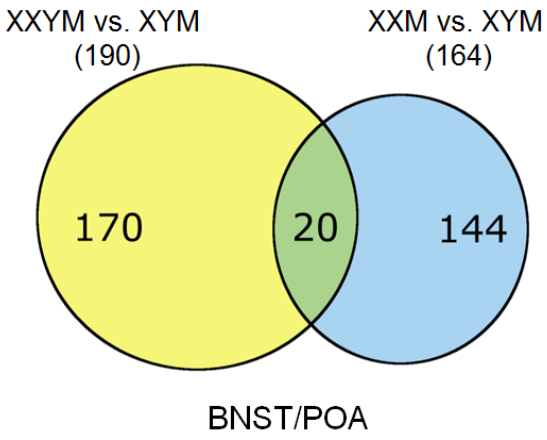


Table 2-1: Primers used in the quantitative RT-PCR validation of microarray results.

Gene	Forward primer	Reverse primer	Product size (bp)
GAPDH	TGCCGCCTGGAGAAACC	CCCTCAGATGCCTGCTTCAC	65
Eif2s3x	TTGTGCCGAGCTGACAGAATGG	CGACAGGGAGCCTATGTTGACCA	198
Kdm6a	CCAATCCCCGCAGAGCTTACCT	TTGCTCGGAGCTGTTCCAAGTG	166

Table 2-2: Median time spent with each stimulus animal in seconds. The interquartile range is given in parentheses. Total length of time of each test is 300 seconds.

Genotype	n	Time spent with stimulus estrus female (s)	Time spent with stimulus male (s)
XYM	13	143 (71.0)	90 (47.0)
XXM	8	121 (65.0)	118.5 (27.8)
XXYM	8	111 (46.5)	140 (63.5)

Table 2-3: Median number of approaches to the stimulus animals. Interquartile range is given in parentheses. The number of times the test animal crossed a line into the incentive zone beginning 5 inches away from the stimulus animal's chamber was counted.

Genotype	n	No. of approaches to stimulus female	No. of approaches to stimulus male	Total no. of approaches
XYM	13	7 (3.5)	7 (3.0)	14 (5.5)
XXM	8	7 (2.5)	8.5 (4.5)	15.5 (5.5)
XXYM	8	7.5 (5.5)	7.5 (4.0)	14.5 (7.5)

Table 2-4: Top 10 pathways that are significantly affected by being XXY in the BNST/POA (p<0.05 Fisher's exact test) as determined by Ingenuity Pathway Analysis.

The list of genes that differ significantly between XXY and XY males was entered into IPA. The 'Ratio' column is the fraction of genes in the input list that are found in that pathway.

No.	Ingenuity Canonical Pathway	p-value	Ratio
1	Complement System	3.89E-03	0.086
2	Antigen Presentation Pathway	5.01E-03	0.075
3	Autoimmune Thyroid Disease Signaling	9.33E-03	0.049
4	Graft-versus-Host Disease Signaling	9.33E-03	0.060
5	L-glutamine Biosynthesis II (tRNA-dependent)	9.55E-03	0.091
6	Granzyme B Signaling	1.02E-02	0.125
7	Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	1.38E-02	0.039
8	Allograft Rejection Signaling	1.45E-02	0.035
9	IL-4 Signaling	3.24E-02	0.038
10	B Cell Development	3.39E-02	0.061

Table 2-5: Genes that differ in both XXYM vs. XYM and XXM vs. XYM in the BNST/POA.

This corresponds to the overlap region (green) in Fig 2-5A. All genes had the same direction of change and most had similar fold-change values between the two comparisons (XXYM vs. XYM, XXM vs XYM).

Gene symbol	Accession	Chr	Fold change (XXYM/XYM)	Fold change (XXM/XYM)
AI314976	NM_207219.2	17	0.8	0.8
Ap2b1	NM_001035854.2	11	0.8	0.7
Atp7a	NM_009726.3	X	0.7	0.8
Camkk2	NM_145358.1	5	0.7	0.8
Ccndbp1	NM_010761.2	2	0.8	0.8
Cdh11	NM_009866.2	8	0.7	0.7
Echdc1	NM_025855	10	0.7	0.7
Eif2s3x	NM_012010.3	X	1.4	1.4
Erdr1	NM_133362.2	X and Y	2.5	4.9
Gm13212	NM_001013808.1	4	1.3	1.4
Gtf2ird2	NM_053266.1	5	1.3	1.4
Jam3	NM_023277.3	9	0.7	0.8
Kcnq2	NM_001006677.1	2	0.7	0.7
Kdm6a	NM_009483.1	X	1.3	1.3
Mid1	NM_183151.1	X	3.7	2.4
Myo9a	NM_173018.2	9	0.8	0.8
Slc5a5	NM_053248.1	8	0.6	0.6
Spag9	NM_001025430.1	11	0.7	0.7
Xist	NR_001463.2	X	2.3	9.2
Zbtb46	NM_027656.2	2	1.2	1.3

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Chapter 3

The organizational effect of testosterone on the methylome of the BNST/POA is late-emerging and dynamic

Introduction

The biological basis of sex differences in the brain has been the subject of many recent studies. Numerous neurological diseases (e.g., autism, schizophrenia, Parkinson's disease, etc.) show sexual dimorphism in prevalence [1-5] and studying the effects of sex-specific factors may provide clues about neural health and development. Great advances have been made in the field of brain sexual differentiation, underscoring the role of sex steroid hormones such as testosterone (T) during sexually dimorphic brain development [6, 7]. Testosterone secretion from the gonads and its aromatization to estradiol in the brain at a certain perinatal time window (referred to as the sensitive period) leads to long lasting and irreversible organizational changes that could ultimately determine the fate of the brain with respect to masculinization or feminization [8]. Therefore, it is not surprising that a great deal of effort has been made in understanding the organizational effects of gonadal secretions leading to the dogma that gonadal hormones are the main causative agents of brain sexual differentiation [6, 9-13].

In addition, emerging evidence now suggests that the sex chromosomes carry genes that could influence neurodevelopment, brain function, and behavior. These genetic effects can be independent of or in concert with gonadal secretions and are termed direct genetic effects [14]. They also play an important role in shaping sex differences in brain and behavior [15-17].

Despite much progress in understanding the cellular mechanisms underlying the hormonal regulation of brain sexual differentiation, surprisingly little is known or understood about many of the fundamental molecular mechanisms. We hypothesized that

long term effects of hormones in producing brain sex differences may involve epigenetic modifications such as DNA methylation. Methylation is the addition of a methyl group to carbon-5 of a cytosine located 5' to a guanine nucleotide, the CpG dinucleotide motif. Dynamic regulation of 5-methylcytosine (5-mC) marks at CpG islands in gene promoters is known to affect gene transcription [18, 19], inactivate or activate endogenous transposable elements [20], modulate X-inactivation and imprinting, and regulate heterochromatin in centromeres and telomeres [21]. Emerging evidence implicates epigenetic mechanisms as important players in activity-dependent nervous system functions (e.g., synaptic plasticity, adult neurogenesis, learning and memory, addiction, circadian rhythm, and neuronal plasticity). Forebrain-specific Dnmt1 and Dnmt3a knockout mice show impairments in neuronal morphology, synaptic plasticity, learning and memory. The involvement of Dnmt3a in emotional behavior and spine plasticity in adult mouse nucleus accumbens has also been documented. Recent studies have identified several specific CpGs that could be modified upon sex steroid hormone supplementation during the neonatal period. For example, estradiol can alter the DNA methylation status of certain CpG sites along the estrogen receptor α (ERα), estrogen receptor β (ERβ) and progesterone receptor (PR) promoters [22]. These genes are known to play an essential role in the development of sexually dimorphic brain regions and their proper regulation is critical to the process of sexual differentiation. However, most these investigations have only examined the methylation status of a limited number of CpG sites within specific genes and thus larger-scale studies of the epigenome may provide further insight into the effects of epigenetic modifications on sexual organization of the brain.

Therefore, in order to understand the scope and overall properties of hormone-induced changes in neuronal DNA methylation, we analyzed the methylomes of male (XY), female (XX), and female mice that had been treated with testosterone (XX + T) on the day of birth. Methylation profiling was carried out for a region that encompasses both the bed nucleus of the stria terminalis and preoptic area (BNST/POA), which is sexually dimorphic. This was done at two different time points: postnatal day (PN) 4, which is during the sensitive period and PN60, which is during adulthood. Using reduced representation bisulfite sequencing (RRBS), which enables genome-wide profiling of the DNA methylome at single base resolution, we generated, to the best of our knowledge, the first global map of 5-mC during development and adulthood. In addition, we examined the effect of age and assessed whether developmental stage effects on the DNA methylation landscape shows sex-specific changes. Lastly, we sought to examine whether methylation state differences are reflected in the gene expression patterns.

Here, we show that many changes in CpG methylation status occur in response to testosterone, particularly during adulthood. Interestingly, testosterone induces a shift in DNA methylation from a female-typical to a more male-typical pattern at multiple loci by day 60 of life. Contrary to our expectations, the shift toward male values is only observed during adulthood. Our analysis also demonstrates that a subset of genes which display differential methylation due to testosterone have similar methylation levels between males and females suggesting that testosterone may prevent, as well as induce, brain sex differences. This study demonstrates for the first time how the DNA methylation landscape of the neonatal mouse BNST/POA is altered in response to steroid hormones such as testosterone and implicates a role for DNA methylation in brain sexual differentiation.

Materials and methods

Animals and neonatal injections

All studies were approved by the University of California, Los Angeles (UCLA) Committee on Animal Research. C57BL/6J female and male mice were purchased from Jackson Laboratories (Bar Harbor, ME) and housed at the UCLA Animal Care Facility. Animals were maintained at 20°C with a 12-hour light/12-hour dark cycle, provided ad libitum food and water, and allowed to acclimate for 1 week before initiation of experiments. This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Female mice were mated and once pregnant, cages were checked periodically for pups. On the day of birth, PN0, C56BL/6J male pups were treated subcutaneously with 15 µl of sesame oil (vehicle); female pups were either treated subcutaneously with 15 µl oil (vehicle) or with 100 µg testosterone propionate (Sigma-Aldrich, St. Louis, MO) in 15 µ oil, a dose resulting in clear sparing of the levator ani muscle in testosterone treated females (data not shown). These mice were gonadectomized at 21 days of age before puberty and implanted with a 5-mm-long Silastic capsule (inner diameter: 1.57mm; outer diameter: 2.41mm) filled with testosterone at PN45 to eliminate the potential confound of circulating hormone effects. Animals were then euthanized, and the striatum and BNST/POA were collected at PN4 or PN60.

RRBS library construction

5µg of mouse DNA isolated from the striatum and BNST/POA was digested at 37 °C overnight with 200U of MspI (Fermentas), a methylation-insensitive restriction enzyme

that selectively cleaves the sequence C′CGG and enriches for CpG rich regions, such as CpG islands, promoter regions, and enhancer elements.

Purified restriction fragments were phenol-chloroform purified, end repaired, and adenylated in a reaction containing 20U Klenow exo$^-$ (NEB) and premixed nucleotide triphosphates (1mM dGTP, 10mM dATP, 1mM 5′ methylated dCTP). The reaction was incubated at 30 °C for 30 min followed by 37 °C for additional 30 min. Adenylated DNA fragments were ligated with preannealed 5-methylcytosine containing Illumina adapters in a 20µl reaction made of 1µl Quick T4 DNA ligase (NEB), 1–2µl of 15µM adapters at 25 °C for 15 min. Premethylated adaptors were used to ensure that the cytosines were not affected during the bisulfite reaction. MspI-digested, Illumina adaptor-ligated samples were ultimately size selected, denatured, and treated with bisulfite. For each sample, fragments that were between 120 and 220 bp in size were excised from a 2% Low Range Ultra Agarose gel (Biorad) and stained with SYBR Gold (Invitrogen). QIAquick (Qiagen) cleaned-up fragments were bisulfite treated using the CpGenome DNA Modification Kit from Millipore. Analytical 10µl PCR reactions containing 2µl of bisulfite-treated DNA, 0.2µl each of 10µM genomic PCR primers 1.1 and 2.1 (Illumina) and 5µl MyTaq HS Red 2x Mix (Bioline) were set up to determine the optimal cycle number. The final library was then synthesized by amplification of the bisulfite converted DNA using the determined PCR profile: 2 min at 98 °C, *n* X (30 s at 94 °C, 30 s at 65 °C, 30 s at 72 °C), 5 min at 72 °C, with *n* being the optimal cycle number for each sample. Libraries were purified and sequenced using Illumina HiSeq 2000.

Alignments of reads and data analysis

Reads were called using a standard software and aligned against the two sets of *in silico* reference sequences of all the predicted MspI restriction fragments (one unconverted and one bisulfite converted version). Reads were subsequently mapped back to these reference sequences and C-T mismatches (in cases where a C in the read is matched to a T in the converted reference) were counted for methylation analysis. The methylation level of a C base was calculated as shown here:

(# reads containing a C-T mismatch) / (# reads at that position)

The overall methylation status of a particular locus can be calculated as the average methylation along all of its CpGs [23]. Differential methylation was determined for fragments containing a minimum of 3 CpGs common to all samples by calculating t_i for each of the Cs in the fragment (the t score from the Student's t test). Then, methylation levels between the two groups were compared and the z score of the average t score was estimated as a measure of the differential methylation within this fragment. A fragment was considered differentially methylated if: (i) the mean methylation levels in the two groups differed by at least 10%; and (ii) the z score corresponded to the false discovery rate (FDR) of less than 10%. A gene was deemed differentially methylated if: (i) it overlapped with any of these differentially methylated fragments; or (ii) its transcription start site was within 5Kbp of the fragments.

Gene Ontology using Ingenuity Pathway Analysis

Functional analysis of statistically significant DNA methylation changes was performed with Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Ingenuity functional analysis identified networks, canonical signaling pathways, and biological

functions and/or diseases that were most significantly affected by testosterone, non-testosterone factors and age. For all analyses, data sets containing gene identifiers and corresponding delta methylation values were uploaded into IPA. For network generation, each identifier was mapped to its corresponding object in the Ingenuity® Knowledge Base. These molecules, called Network Eligible molecules, were overlaid onto a global molecular network developed from information contained in the Ingenuity Knowledge Base. Networks of Network Eligible Molecules were then algorithmically generated based on their connectivity. To identify biological functions and diseases that were enriched in the different data sets, genes were associated with biological functions and/or diseases in the Ingenuity Knowledge Base. Right-tailed Fisher's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that data set is due to chance alone. Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured in 2 ways: i) a ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. ii) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone.

Testosterone Measurements

Samples were collected at the time of euthanasia. In all cases, blood was obtained from the carotid artery following decapitation. Blood samples were then processed to isolate serum

and stored at -20C until assays for testosterone were performed. Testosterone assays using radioimmunoassay were performed by Ligand Assay and Analysis Core at the University of Virginia Center for Research in Reproduction (supported by NICHD (SCCPIR) Grant U54-HD28934). Testosterone measurements were performed in singlet reactions using Siemens Medical Solutions Diagnostics testosterone RIA with a reportable range of 47.3-170.5 ng/L. There were no significant differences in measured testosterone levels between our experimental groups using the Kruskal-Wallis one-way analysis of variance test ($H=3.8$, 2 d.f., $p=0.15$).

Determination of testosterone-induced masculinization

To assess whether testosterone can induce a broad shift in DNA methylation in the brain of XX + T mice from a female-typical to a more male-typical pattern, we first identified CpG sites that were sexually dimorphic and defined them as those i) that had a difference of at least 15% in methylation levels between control females and control males; and ii) that were significantly different ($p < 0.05$ measured by the Student's t-test). This analysis identified about 10,000 sites (FDR ~7 to 13%) in each brain region. For each site, we defined the male methylation level as 0 and the female level as 100. The methylation level in females treated with testosterone at the sites was renormalized to this scale and graphed on a continuum between 0 and 100 (i.e., the rescaled male and female methylation levels).

Results

Sex effects on genome wide methylation data

Sex-specific changes in brain DNA methylation are not well understood. To address this issue, we first compared genome-wide maps of 5-mC in adult mouse BNST/POA in both XX and XY mice using reduced representation bisulfite sequencing (RRBS). RRBS is a well-established technique for sequencing the DNA that has been digested with MspI, a methylation-insensitive restriction enzyme that recognizes CpG-rich sites in the genome. From two biological replicates (each of which consisted of a pool of three animals) per age, brain region, and sex for a total of 24 samples, we generated on average a total of ~125 million uniquely mapped reads (Supp. Table 3-1) for each biological condition. CpG sites that were not present in all comparison groups were excluded from further analysis. We interrogated 1.39 million CpG sites, reflecting ~3.8% of all CpGs in the mouse genome.

Assessment of genome-scale patterns of 5-mC indicated that overall methylation profiles of adult XX and XY were highly similar across all chromosomes (*Pearson coefficient*, 0.99) (Supp. Fig 3-1). These data indicate that the genomic profiles of 5-mC were both reproducible and highly similar for both sexes. Despite overall similarity, hierarchical clustering clearly reflected sex-specific 5-mc dynamics (Fig 3-1A). In addition, developmental stage-dependent loci (Fig 3-1C-E) methylation differences were present. Together, these data suggest that DNA methylation can regulate tissue-, sex-, and developmental stage-specific programs in the brain.

To identify genes that undergo sex-specific methylation, we compared the methylomes of adult male (XY) and female (XX) mice and found a large number of genes that showed sex differences in methylation patterns (1029). As a validation of our approach, we then analyzed X-chromosome linked genes. Because of the process of X

inactivation in female mice, we expected to find hypermethylation in substantially more genes in females compared to males. Our findings were consistent with this hypothesis. 426 of the 1029 sex-affected genes were X-linked and 359 (84.27%) were more methylated in females (Fig 3-2).

Focusing on the effect of sex on autosomes, we identified 600 genes showing sex differences in the adult BNST/POA. Interestingly, a substantial number of these genes showed higher methylation in males than females 520/600 genes were more methylated in males relative to females during adulthood (Fig 3-2). Together, these data reinforce the idea that sex-specific regulation of 5-mC status occurs in the brain.

Testosterone-induced modification of brain DNA methylation

Sex differences in methylation patterns can be attributed to discrete sex hormones produced by the two sexes (e.g., testosterone and/or estradiol) and other influences (e.g., direct genetic effects). Testosterone- and estradiol-induced changes in DNA methylation levels at particular CpG sites on specific promoters have been reported in association with sexual differentiation of the brain [24]. Here, we sought to determine the effects of neonatal testosterone exposure on DNA methylation *genome-wide*. To examine the testosterone-dependent dynamics of 5-mC, we subjected female mice to a dose of testosterone that had been previously shown to be masculinizing on the day of birth and compared the DNA methylation status of female (XX) mice, and female mice treated with testosterone (XX + T). We found that a substantial number of differentially methylated fragments were modified by testosterone. These fragments mapped to a relatively small number of genes at PN4 (45 genes). By day 60 of life, a much larger number of genes demonstrated methylation

changes in response to testosterone (740 genes) (Fig 3-3A and Supp. Table 3-2). 36% of testosterone-affected genes exhibited sexually dimorphic methylation patterns (265/740). We also detected a subset of genes with similar methylation levels between males and females that displayed differential 5-mC levels in XX + T in response to testosterone administration. 475/740 (64%) of testosterone-affected genes in the PN60 BNST/POA showed no sex-specific methylation differences (Supp. Table 3-3). These data suggest that in certain contexts, testosterone may prevent – and not just induce – sex differences in DNA methylation. Such effects on DNA methylation may serve to compensate for differences between the two sexes to keep them as equivalent as possible, particularly in those genes that have comparable functions in males and females [25].

Further assessment of 5-mC levels at testosterone-affected genes demonstrated that a substantial fraction display increased 5-mC in response to testosterone. 38/45 (84.44%) genes at PN4 and 705/740 (95.27%) genes at PN60 also showed greater methylation in female mice treated with testosterone (Fig 3-3B). Testosterone effects on chromatin modifying genes were observed in the BNST/POA. Methylation at *Ctbp1* (a transcriptional repressor that interacts with histone deacetylase 1) is significantly increased in XX+T at PN4 [26]. On the other hand, methylation at *Msl3* (which is part of the MSL complex involved in the spreading of histone H4 lysine 16 acetylation) is reduced in PN60 XX+T animals [27, 28]. These data suggest that one mechanism by which hormones induce brain sexual differentiation could be regulation of the methylation of genes that are part of the epigenetic machinery. One likely hypothesis is that these genes are acting as 'master regulator' genes and can help explain the increase in the number of differentially methylated genes seen at PN60.

Several genes related to cell survival and death were found in the testosterone-affected dataset at PN4. Recent studies show that testosterone exposure leads to differential apoptotic rates which is at least partially responsible for the establishment of the sexually dimorphic nuclei in this region [29]. Here, *Bcor* was more methylated in XX+T. The protein product of this gene is a corepressor of *Bcl-6* and is found with it at known Bcl-6 targets, including several regulators of cellular proliferation and apoptosis [30]. Additionally, Bcor interacts with class I and II histone deacetylases suggesting that it achieves transcriptional silencing using these components of epigenetic machinery [31]. A second apoptosis-related gene, *Commd1*, also appeared to be more methylated in the BNST/POA dataset. *Commd1* increases ubiquitination and therefore degradation of NF-κB, which plays an important role in the Bcl-2/Bax cell death pathway which has been shown to play a critical role in establishing the sexual dimorphisms seen in the BNST and POA [32, 33].

To examine the characteristics of testosterone-affected genes in adulthood, we used the Ingenuity Pathways Analysis software (Ingenuity® Systems, www.ingenuity.com) on the list of genes obtained from the BNST/POA. Our analysis revealed that testosterone alters the methylation of genes belonging to a wide range of biological processes and functions (Supp. Table 3-4). Table 3-1 lists some of the functional categories that were enriched at PN60. Functional categories related to nervous system development were strongly represented in the testosterone-affected dataset at PN60 (p-value range: 4.71E-08 to 1.93E-02). Many of these functional categories represented basic processes that are crucial for general neural function (for example, morphology of nervous tissue, neuritogenesis, guidance of axons, and morphology of dendritic spines). Genes related to

biological processes such as organization of cytoskeleton, microtubule dynamics, and apoptosis were also altered by testosterone at this time point.

Other functional categories enriched in the BNST/POA included quantity of neurons, cell viability of neurons, and proliferation of neuronal cells (Table 3-1). The presence of genes related to expansion and survival of neurons in the testosterone-affected dataset is intriguing given recent evidence that cell birth may be an important mechanism that helps maintain the sexual dimorphism in the sexually dimorphic nucleus of the POA [34]. As mentioned earlier, the establishment of the sexually dimorphic nuclei in the BNST and the POA is reliant on highly similar cell death mechanisms [35, 36]. Therefore, our data suggest that testosterone-influenced cell addition may be another common feature of these sexually dimorphic brain regions and, more generally, may be a widespread mechanism by which sexual differentiation of the brain is maintained. Although the involvement of testosterone-affected genes in both cell death and cell birth may appear to be contradictory at first, we hypothesize that testosterone is shutting down cell death genes but affecting the methylation patterns at cell birth genes to activate their expression.

Identification of stably differentially methylated genes

We examined the stability or loss of 5-mC at genomic locations that were shown to be affected by testosterone in the neonatal brain. Direct comparison of testosterone-regulated sets of genes between PN4 and PN60 allowed us to separate dynamic from stable differentially methylated genes. We found 11 genes whose magnitude and/or level of DNA methylation changes between XX and XX +T were similar in PN4 and PN60 and classified them as stable (Table 3-2).

Notably, several distinct effects of neonatal testosterone were apparent on gene methylation levels. For 4 genes, the direction and the magnitude of the neonatal testosterone effect on methylation was still present at the same genomic location at PN60, while for 7 genes the neonatal testosterone effects reemerged at a different location of the gene during adulthood. Unexpectedly, for 2 genes the testosterone-dependent epigenetic modifications were associated with a reversal of direction from demethylation neonatally to increased methylation later in life or vice versa. Altogether, these data suggest that 5-mC regulation might be more dynamic than we had originally predicted (Table 3-2).

Testosterone-induced masculinization of methylation

Considering that we found testosterone-influenced gene methylation in discrete brain regions of the developing brain, we sought to assess whether testosterone can induce a broad shift in DNA methylation in the PN4 and PN60 brain of XX + T mice from a female-typical to a more male-typical pattern. We first identified CpG sites that were sexually dimorphic and defined them as those that: i) had a difference of at least 15% in methylation levels between control females and control males; and ii) were significantly different ($p < 0.05$ measured by the Student's t-test). This analysis identified about 10,000 sites at each age (FDR ~7 to 13%). For each site, we defined the male methylation level as 0 and the female level as 100. The methylation level in females treated with testosterone at the sites that displayed sex differences was renormalized to this scale and graphed on a continuum between 0 and 100, the rescaled male and female methylation levels, respectively. Schematic representations of several hypothetical scenarios by which testosterone affects CpG methylation are depicted in Fig 3-4.

When we plotted histograms of where these sites in XX+T fall at each age, almost all the sexually dimorphic CpG sites were more female-like in the XX+T group at PN4 (Fig 3-5A). By day 60 of life, a number of sexually dimorphic CpG sites BNST/POA demonstrated methylation levels more similar to males than to females although the majority remained female-like (Fig 3-5B). These data imply that there is a dramatic change in the distribution of sexually dimorphic molecular marks along the female to male spectrum as the animal ages resulting from the organizational effects of testosterone.

Non-testosterone mediated effects

Sex steroid hormones, while important, are not the only factors responsible for brain sexual differentiation. Certain sex differences are formed independent from the gonads. Direct genetic effects mediated through genes encoded by the sex chromosomes may also underlie the sexual organization of the brain. To tease apart testosterone-independent effects on DNA methylation, I first identified genes that showed a difference of at least 10% in methylation levels between control females (XX♀) and control males (XY♂) (FDR < 10%). I excluded genes from this list if their methylation was shown to be influenced by testosterone. I also did not consider those X-linked genes that showed higher methylation in females than in males were also not considered because of the special involvement of DNA methylation in dosage compensation. In the neonatal brain, I identified 253 testosterone-independent, sex-specific differentially methylated genes in the BNST/POA whereas in the adults, I found 516 genes (Supp. Table 3-5).

Among the genes where DNA methylation was affected by non-testosterone factors at PN4 was *Atrx*, which is a DNA helicase. ATRX interacts with MeCP2 and disruption of this

interaction has been linked to cases of inherited mental retardation, which is male-biased [37]. In adulthood, one such gene was *Kiss1*. This gene is critically involved in the regulation of the estrous cycle and is expressed in a sexually dimorphic manner in the anteroventral periventricular nucleus of the hypothalamus (AVPV) due to the perinatal organizing effects of testosterone [38, 39]. Methylation at several CpGs is sexually dimorphic in the AVPV although the relative contribution of hormonal vs. non-hormonal factors is unknown [40]. Thus our data indicate an involvement of non-hormonal factors in establishing this sexual dimorphism. Complete IPA lists of canonical pathways, biological functions, and networks are shown in Supp. Table 3-6.

Hormonal and non-hormonal regulation of the establishment and maintenance of sexual dimorphism in the BNST/POA

Apoptosis-related genes were significantly enriched in the set of genes affected by perinatal testosterone exposure in PN60 animals. This enrichment was not found in the set of genes affected by non-testosterone dataset although there was overlap between the two datasets in numerous functional categories important to the development of the nervous system (e.g. guidance of axons, morphology of nervous system, neuritogenesis). This indicates that the sexually differentiated apoptotic rates seen in this brain region (which appear to *establish* the sexual dimorphism in volume in the BNST and POA) is mostly under the control of gonadal hormones and that other factors (such as direct genetic effects) play only a small role in regulating this process.

One of the functional categories enriched in both the testosterone and non-testosterone comparison sets is proliferation of neuronal cells (Fig 3-6). Therefore, the

maintenance of the sexual dimorphism in volume (which is due at least in part to pubertal cell birth) may be controlled by both hormonal and non-hormonal elements. The number of genes associated with this category was similar in both datasets (13 in the testosterone group and 11 in the non-testosterone one) but only 2 genes were found in common (*Hhip* and *Znf423*). Thus, it appears that the two factors may control different parts of the cell birth machinery. This may allow more modular control over the maintenance of sexual dimorphism in the BNST/POA.

Developmental age-specific effects

To determine the effect of age on methylation levels, we compared the methylation profile of mouse striatum and BNST/POA during PN4 with that of PN60. Quantification of global DNA methylation levels showed that overall, DNA methylation increases with age in mice (p-value < 0.005) (Fig 3-7). This analysis revealed that methylation patterns of 3316 genes was altered between PN4 and adulthood irrespective of genotype or treatment (Supp. Table 3-7). We then analyzed the enrichment of functional categories in these age-affected genes using IPA. In the context of nervous system function, the most significantly enriched functional categories were related to the morphology of the nervous system, neuritogenesis (a detailed breakdown is shown in Supp. Table 3-8).

In addition, we identified a number of age-related statistically significant signaling pathways unique to each sex. Although there were a large number of genes that were affected by age in just one sex, many of the signaling pathways associated with the genes in these two datasets were shared between the sexes. These pathways include those that were essential to basic nervous system function and development such as axonal guidance

signaling, synaptic long term potentiation, and GNRH signaling. XX-specific age-affected pathways included prolactin signaling, neuregulin signaling, and neural growth factor signaling. Pathways unique to males included glutamate receptor signaling, Notch signaling, and Ephrin B signaling (Supp. Table 3-9).

Discussion

Our study shows how testosterone can modify the DNA methylation landscape of the brain. In contrast to previous studies that have only focused on the effect of early hormone exposure on the DNA methylation status of a limited number of genes – particularly hormone receptors – we have established the first genome-wide and quantitative map of testosterone-induced CpG methylation changes in the BNST/POA. To demonstrate the effects of neonatal testosterone exposure on the epigenetic DNA landscape, we compared the methylation maps of male, female, and females treated with testosterone during the critical period and in adulthood. We found that the methylation patterns of a large number of genes differed between the sexes. In addition, a marked enrichment of DNA methylation was observed in females, consistent with the phenomenon of X chromosome inactivation. Moreover, testosterone altered the methylation status of a large number of CpGs particularly in the adult brain. My further assessment of methylation demonstrated that a substantial fraction also displayed non-testosterone mediated effects suggesting the involvement of direct genetic effects.

This study revealed several key aspects of testosterone-induced epigenetic DNA modifications. First, we found that during the critical period, there was very little testosterone influence on methylation levels. This number increased dramatically in

adulthood and an appreciable subset of sexually dimorphic CpG sites were masculinized in response to testosterone during this time point. These results ran counter to our initial hypothesis. Instead of establishing methyl marks during the perinatal period that persist into adulthood, the molecular effects of testosterone organization appear much later in life and appear to be very dynamic. This finding implies that the emergence of certain sex differences in the brain may be a gradual process that is cemented over the organism's life. Notably, we also identified sets of testosterone-regulated loci that clearly maintained 5-mC from PN4 to 60 although these were a small minority of the overall testosterone-affected dataset. The overwhelming majority of testosterone-affected loci showed dynamic DNA methylation patterns. While this is not in agreement with the prevailing view of DNA methylation as permanent epigenetic mark, our data was consistent with the findings of Schwarz et al. where they observed that sex differences in methylation patterns at the ERα, ERβ and PR promoters were dynamic across the life span [22]. However, in our data the methylation patterns of these three promoters were not significantly influenced by sex or testosterone exposure. This was not unexpected as they only detected modest differences between their experimental groups. The maximum degree of difference that they found (~8%) was less than the cutoff that we used to determine differentially methylated fragments (10%). Additionally, we required our differentially methylated fragments to show consistent methylation changes in several adjacent CpG sites whereas they focused on single site differences. Altogether, our data provide a new perspective on the mechanisms underlying organizational effects of hormones. Contrary to the expectation that adult brain sex differences are formed within the first few days after birth, which then persist into adulthood; we find that organizational effects of hormones on molecular

markers including DNA methylation are not immediately evident but emerge over a longer time scale.

When we examined the characteristics of the genes associated with the testosterone-modified CpGs, the altered genes were significantly enriched in genes that are expressed in the brain. Testosterone-modified CpGs were also associated with chromatin modifying genes, suggesting that one mechanism by which testosterone induces brain sexual differentiation is by modifying the methylation status of genes that are part of the epigenetic machinery. Functional analysis revealed significant over-representations of genes involved in synaptic function.

In the BNST/POA, sexually differentiated rates of apoptosis (female>male) driven by testosterone exposure is one of the major events leading to the sexually dimorphic nuclei in this region. Consistent with this, we found genes involved in apoptosis in the testosterone-affected dataset at both PN4 and PN60. For instance, *Bcl2* becomes hypermethylated in response to testosterone at day 60. The Bcl-2/Bax pathway has been demonstrated to play a crucial role in testosterone-modulated apoptosis [35, 36]. There was also hypermethylation driven by testosterone at the pro-survival genes *Gdnf*, *Xiap*, *Flt1*, and *NTRK2* (receptor for Bdnf) in adulthood [41-44]. Perhaps most intriguing, however, was the testosterone-affected genes related to the proliferation of neuronal cells. It was assumed that once testosterone had organized the brain perinatally, the resulting sexual dimorphism was then passively maintained throughout the animal's life. However, recent evidence has shown that this maintenance may be a more active process than once assumed and requires reinforcement in the form of pubertal hormones [34, 45, 46]. At least

some of this reinforcement appears to take the form of sexually differentiated rates of cell addition, which may be a widespread mechanism as it has been shown to take place in several sexually dimorphic brain regions including the POA and locus coeruleus [34, 47, 48].

However, the pathways involved in this cell birth have not yet been elucidated. Of the 13 testosterone-affected genes found to be related to neuronal proliferation, 5 (*Ankrd6*, *Fzd9*, *Hhip*, *Irx3*, *Vax1*) were not part of the apoptosis group and were all hypermethylated in XX+T vs XX. Aside from *Hhip*, all these genes were heavily involved in the Wnt signaling pathway. *Ankrd6*, or Diversin, expression is strongly associated with areas of active cell proliferation in the brain like the subventricular zone and knockdown of its expression leads decreased proliferation of neuroblasts and promotes the degradation of β-catenin [49, 50]. *Fzd9* is part of the canonical Wnt signaling pathway and appears to play a critical role in patterning the developing telencephalon [51]. It is highly expressed in neural stem cells [52]. *Irx3* is part of the highly conserved *Iroquois* family of homeoproteins which participate in a wide variety of developmental processes [53]. It is expressed in the neural tube and helps specify the identity of the neurons generated here and has been shown to be a direct target of the Wnt/β-catenin pathway [54, 55]. Lastly, *Vax1* is expressed in the developing forebrain and helps regulate the development of the forebrain and the visual system [56]. Taken together, these data suggest that the Wnt signaling pathway may be one of the routes by which testosterone organizes sexually differentiated cell birth.

I also observed a number of genes for which the sex differences in methylation were not due to testosterone. These data hint at the effects of testosterone-independent sex-

specific mechanisms of brain sexual differentiation. There is now clear evidence that genes encoded by the sex chromosomes exert direct sex-specific effects on the brain. For example, the testis-determining gene *Sry* is expressed male-specifically in nigral neurons that send dopamine projections to the striatum. The overall outcome of dopamine release in striatum is facilitation of movement, regulation of synaptic plasticity critical in autonomous learning (implicit memory), and coding reward and motivational behavior that play a role in addiction. Interestingly, when the expression of *Sry* is transiently downregulated in dopamine neurons of substantia nigra in male rats, levels of tyrosine hydroxylase (TH)—the rate-limiting enzyme in dopamine synthesis—drop in the striatum and rats exhibit movement deficits such as those observed in Parkinson's disease. These data illustrated for the first time that genes encoded on the sex chromosomes can have a direct contribution to brain sexual differentiation.

A striking feature of both testosterone- and non-testosterone-affected datasets in adulthood was that both factors increased methylation at genes related to cell proliferation with the sole exception being *Psen1* (less methylated in response to testosterone). *Psen1* is one of the four major proteins that make up the presenilin complex and plays a critical role in the generation of amyloid β-protein, the accumulation of which is strongly implicated in the onset of Alzheimer's Disease (AD). In fact, mutations in this gene seem to account for the majority of cases of familial AD [43]. Male mice that are hypogonadal express significantly higher levels of proteins related to AD, including *Psen1*, compared to control males [44]. Interestingly, recent evidence suggests links between AD pathology and demethylation at the *Psen1* promoter [45, 49].

With our current study, we bring further evidence for the importance of the testosterone in regulation of methylation. Taken together, our results suggest that early testosterone exposure has broad effects on brain methylation patterns particularly during adulthood. However, it is worthwhile to note that there are limitations to this study. This work only represents a snapshot of DNA methylation landscape while the brain may display a vast array of epigenetic plasticity as it passes through different stages of development including hormonal changes during puberty. Longitudinal study designs examining DNA methylation changes at different life stages can provide a comprehensive picture of the genome and further our understanding of how the epigenome is modified over time. In addition, DNA methylation is associated with other epigenetic alterations, especially histone modifications, and RNAi pathways. Different brain regions also have different epigenetic marks across their genomes. Therefore, studies of other epigenetic changes are crucial to separating the contribution of common mechanisms of epigenetic regulation. Future studies should also focus on mapping the epigenome across multiple tissues. Epigenetic profiling across functionally discrete brain areas will be important in future in identifying sex differences, which serve purposes other than contributing to neuroanatomical differences between the two sexes.

Early hormonal theories of brain sexual differentiation hold that neonatal testosterone immediately establishes sexually dimorphic differences in the brain and that these differences are maintained into adulthood. Our data, on the other hand, provide a new perspective on the mechanisms underlying organizational effects of testosterone. Our studies provide intriguing evidence that sex differences in methylation are not the result of the immediate early actions of testosterone on the brain. Rather, they are induced by

hormonal effects that emerge over time. Clearly, additional studies of genome-scale methylation maps in the future will be important to give us a full understanding of the long lasting influences of early hormone exposure on DNA methylation dynamics of the brain.

FIGURES AND TABLES

Figure 3-1: Heat map of normalized 5-mC based on binned data (10-kb bins) identified by hierarchical clustering. For all heat maps, increasingly darker color represents increased methylation. Chromosomes 12 and X are represented as examples. **A.** Heat map of 5-mC loci in adult XX, XY, and XX + T BNST/POA samples. **B.** Heat map of X chromosome normalized 5-mC in an XX animal. Heat maps of 5-mC loci in PN4 and PN60: **C.** XX, **D.** XY, and **E.** XX + T. Examples of sex- and developmental stage -specific methylation differences are shown in the dashed boxes.

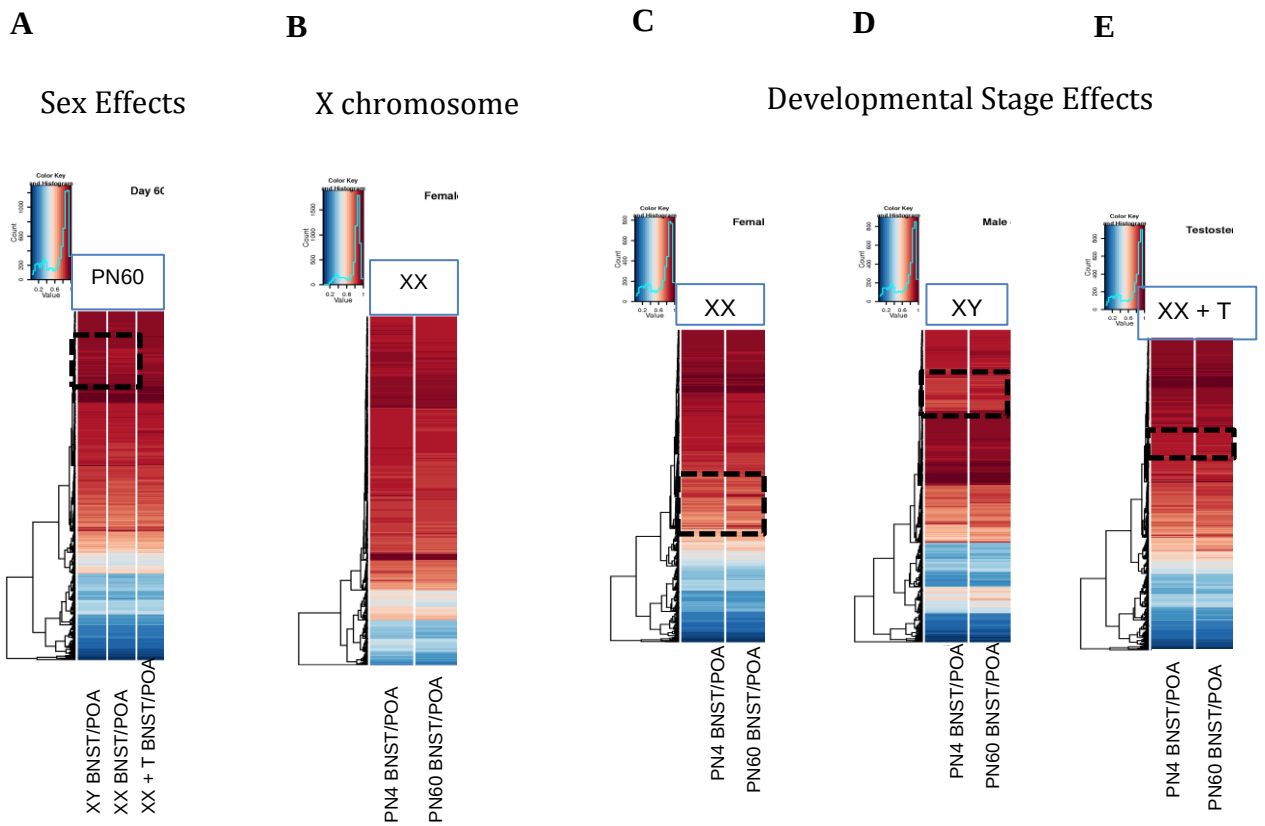


Figure 3-2: Displayed are the fractions of X, Y, and autosomal genes displaying higher methylation in one sex or the other in the PN60 BNST/POA.

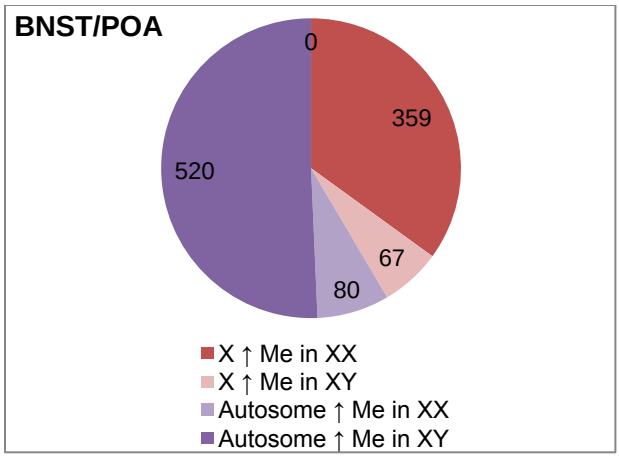
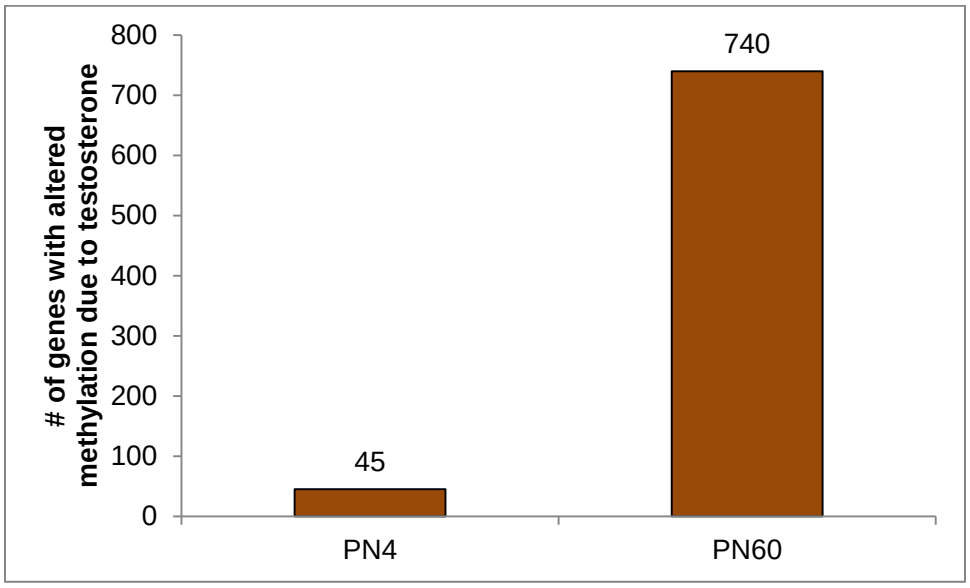


Figure 3-3: The number of genes affected by perinatal testosterone exposure. A. Number of genes where methylation is altered by testosterone in PN4 and PN60. **B.** Fraction of genes that exhibit testosterone-dependent hypo- or hyper-methylation at each age.

A



B

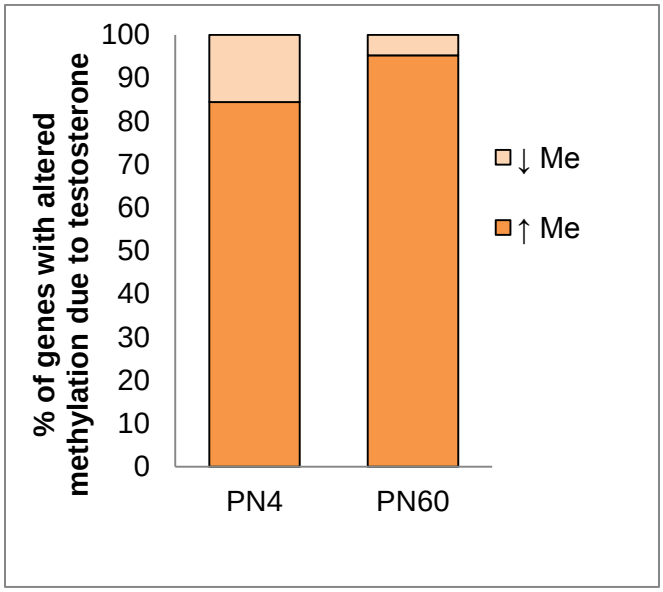


Figure 3-4: Schematic representations of several potential scenarios by which testosterone affects CpG methylation in the brain of female mice treated with testosterone

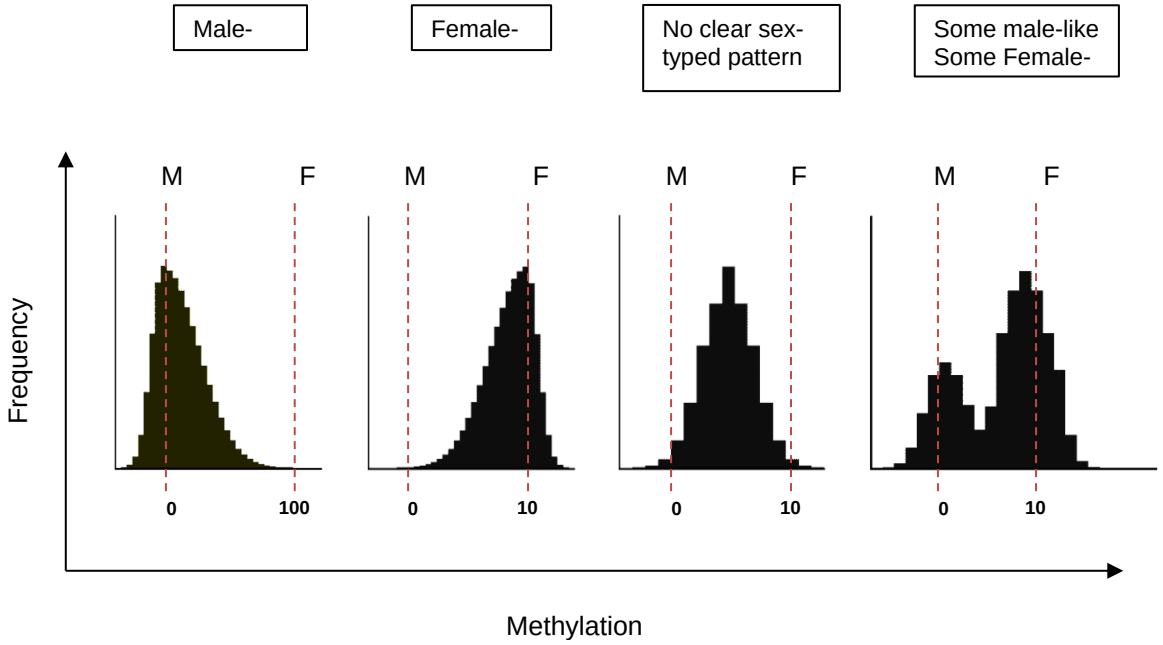


Figure 3-5: DNA methylation patterns are more masculine in XX + T at PN60. Mean XX + T methylation of the genes that display significant basal sex differences (delta methylation$_{(XX-XXY)}$ ≥15%, p value ≤ 0.05) are plotted on a continuum between XY (0) and XX (100) methylation levels in **A.** PN4 and **B.** PN60

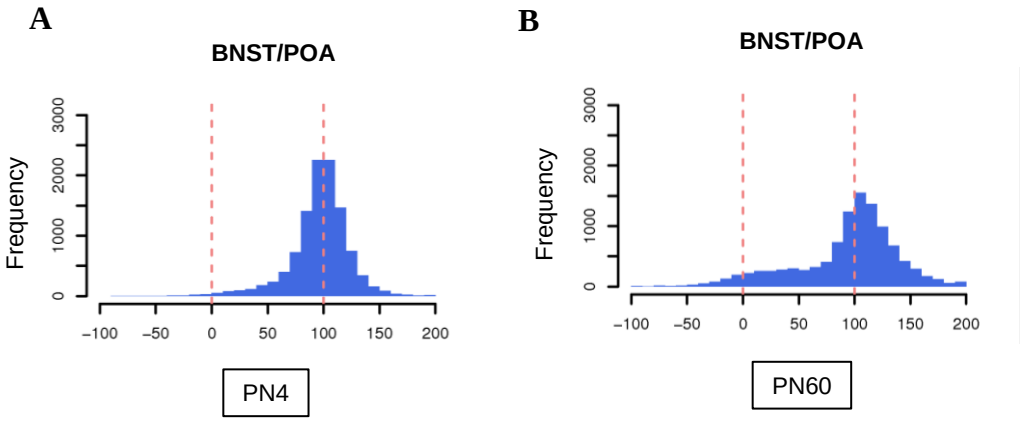


Figure 3-6: Biological functions affected by both testosterone and non-testosterone do not share many genes. Venn diagram showing the number of unique and shared genes between and among the proliferation of neuronal cells functional category associated with the testosterone and non-testosterone dataset in the BNST/POA.

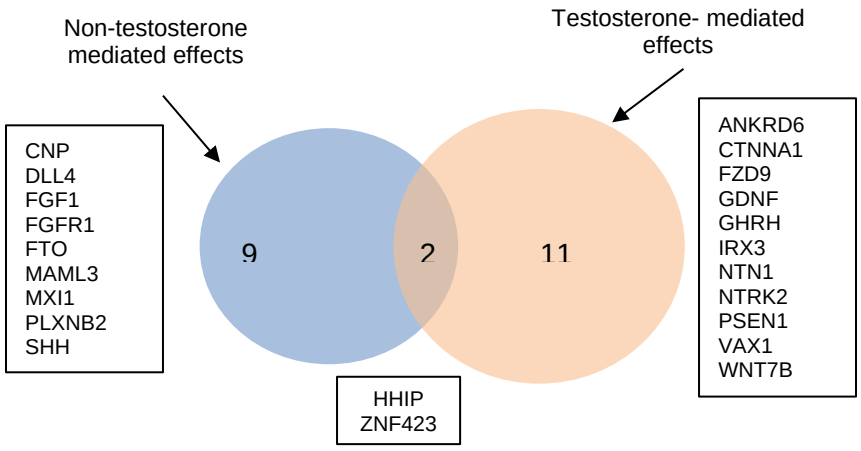


Figure 3-7: Global DNA methylation at each age is represented in this figure. All samples at each age were considered together without regard for experimental group. Statistical analysis was done by student's t test. P-value < 0.05 was considered significant.

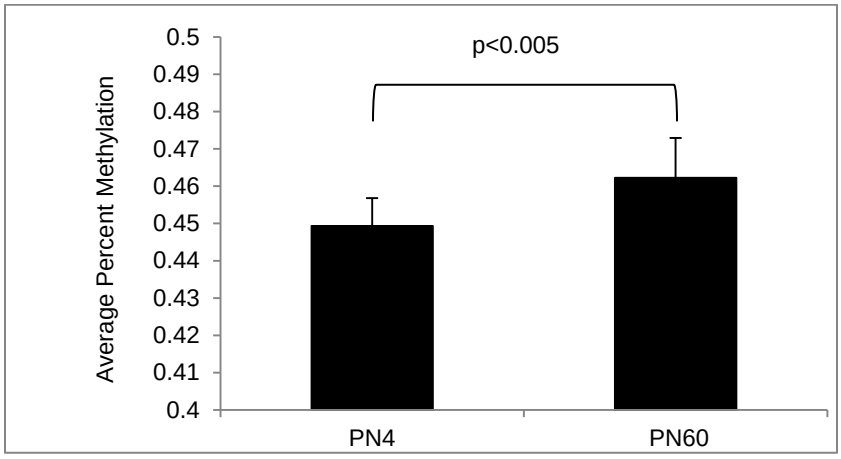


Table 3-1: Examples of functional categories enriched in the testosterone data-set for the BNST/POA.

Category	Function Annotation	BNST/POA	
		p-value	# Genes
Nervous System Development and Function	Morphology of nervous system	3.91E-06	63
	Development of central nervous system	4.71E-08	54
	Morphology of nervous tissue	7.75E-05	44
	Neuritogenesis	1.03E-07	37
	Outgrowth of neurites	2.19E-03	22
	Coordination	1.67E-02	15
	Axonogenesis	1.18E-04	17
	Excitatory postsynaptic potential	5.55E-03	10
	Growth of neurites	5.21E-03	23
	Morphology of neurites	1.99E-04	17
	Morphology of dendritic spines	7.85E-04	4
	Guidance of axons	1.60E-05	18
	Outgrowth of axons	9.82E-03	8
	Quantity of neurons	3.81E-05	24
	Cell viability of neurons	1.93E-02	12
Cellular Assembly and Organization	Organization of cytoskeleton	1.72E-06	77
	Microtubule dynamics	7.64E-06	65
Cell Death and Survival	Apoptosis	1.70E-03	138
Cellular Growth and Proliferation	Proliferation of neuronal cells	1.25E-02	13
Behavior	Learning	2.81E-04	27
	Social behavior	2.59E-03	7

Table 3-2: List of genes where the methylation was stably affected by testosterone.

Negative delta methylation indicates higher methylation in XX + T.

Gene Symbol	Δ Me at PN4	Fragment_Coordinate at PN4	Δ Me at PN60	Fragment_Coordinate at PN60
Igfbp7	-0.13	chr5:77809526-77809779	-0.21	chr5:77786342-77786589
Odz3	-0.11	chr8:49626485-49626755	0.12	chr8:49395012-49395267
Emd	-0.13	chrX:71500275-71500386	-0.11	chrX:71500067-71500242
Herc3	-0.12	chr6:58856760-58856872	-0.12	chr6:58856760-58856872
Commd1	-0.16	chr11:22873668-22873935	-0.12	chr11:22872579-22872758
Bcor	-0.14	chrX:11715730-11715985	-0.11	chrX:11703662-11703859
Nap1l5	-0.12	chr6:58856760-58856872	-0.12	chr6:58856760-58856872
Gpr179	-0.14	chr11:97193837-97194108	-0.11	chr11:97197795-97197992
Zrsr1	-0.16	chr11:22873668-22873935	-0.12	chr11:22872579-22872758
Lonrf3	-0.12	chrX:33868422-33868652	-0.1	chrX:33869078-33869231
Sdk1	-0.13	chr5:142590169-142590448	0.12	chr5:142312410-142312648

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Chapter 4

Conclusion

Sexual differentiation begins from the moment the egg and sperm meet and determine the chromosomal sex of an organism. Although males and females share many similarities, sex differences have been found in numerous aspects of physiology from gonadal development to cardiac function to neural circuits and behavior [1-3]. Traditionally, the process of mammalian sexual differentiation or sexual development has been thought to have two major divisions: (1) sex determination, which is whether the organism develops testes or ovaries; and (2) sex differentiation, which is the development of all non-gonadal sex differences. Sex determination is largely genetically determined and essentially depends on the presence or absence of *Sry*, the testis-determining gene. Sex differentiation, on the other hand, was thought to be the exclusive domain of gonadal hormones and to only occur once sex determination had taken place. This is what we've termed the classical hypothesis and was the lens through which many scientists viewed the study of sex differences.

Given the undeniably strong effects of gonadal hormones, the pervasiveness and persistence of the classical hypothesis is not surprising. Gonadal hormones can have organizational – or long-lasting and irreversible – effects that occur after a single exposure within a defined developmental window and activational – or temporary – effects that are dependent on its presence (for example, testosterone injections inducing mounting behavior in castrated males). This dichotomous view of hormonal action stems from the findings of Phoenix, Goy, Gerall, and Young in 1959 who found that prenatal exposure to testosterone in female guinea pigs masculinizes mating behavior [4]. This was followed by numerous studies demonstrating that exposure to testosterone and its metabolites, such as

estradiol, can act to masculinize distinct regions of the central nervous system and that appeared to translate into attendant masculinization of some sexually dimorphic behaviors in a variety of species, particularly those related to mating [2, 5, 6]. Alongside these findings, there have also been an abundance of studies documenting sex differences in neuroanatomy and chemistry, cognition and behavior where the origin and significance of the sex difference has yet to be determined (see Chapter 1).

The classical hypothesis has begun to be challenged in recent years. A growing body of evidence suggests that gonadal hormones are not the only significant contributing factor to non-gonadal sex differences and that these sex differences can emerge even before sex determination [7, 8]. The role of direct genetic effects, which are essentially the actions of gene products located on the sex chromosomes, is becoming increasingly clear in a variety of species although gonadal hormones is unquestionably still the major factor. Much of this new evidence has come for studies of animal models with unusual sex chromosome complements such as the four core genotypes and the Y* mice but those with sex chromosome aneuploidies (like Klinefelter men and Turner women) have also proven invaluable [2, 9]. Therefore, a reevaluation of the importance of sex chromosomes and their impact on the process of sexual development is in order. In Chapter 2 of this dissertation, we examined the effect of the additional X chromosome in mice from the Sex Chromosome Trisomy (SCT) model, which is a novel model of Klinefelter Syndrome (KS).

Genetic influences on sexual differentiation of brain and behavior

KS men are feminized (more female-typical) in a number of phenotypes. In comparison to XY men, their body fat distribution is more feminine, they have a higher risk

of developing several autoimmune diseases (which are usually female-biased), and they exhibit higher rates of homosexual behavior and gender non-conformity [10-12]. However, KS men are not generally feminized and are still phenotypically male on a variety of parameters. In addition to the additional X chromosome, KS men have lower levels of testosterone at puberty, which is now considered to be a second critical window during which gonadal hormones can also exert organizational effects. Disentangling these two factors (the extra X and the lower pubertal testosterone) is extremely difficult in human subjects and to do so experimentally would be unethical. Therefore, XXY males from the SCT model present a unique opportunity to elucidate the biological origins of phenotypes associated with KS.

We investigated the extent of feminization of behavioral and molecular phenotypes in XXY male mice. We chose to study partner preference – which is one of the most sexually dimorphic traits in the animal kingdom – and gene expression in the bed nucleus of the stria terminalis/preoptic area (BNST/POA), a highly sexually differentiated brain region. Consistent with the feminization of partner choice in KS men, we found that XXY males were less attracted to estrus females and seemed to be more attracted to males. This feminization was not observed in XX males suggesting that the feminization of partner preference in XXY males is not due solely to the presence of the additional X chromosome but rather interactions between this chromosome and the Y. Although gene expression in the BNST/POA was not generally feminized, a small but significant proportion of sexually dimorphic genes were found to be more female-typical in their expression patterns. Additionally, we identified a subset of genes where expression was uniquely affected by being XXY. The overlap between these two sets of genes (feminized and XXY-affected),

represent strong candidates for further study to elucidate the molecular basis for KS-specific phenotypes.

An intriguing question concerning the feminization of partner preference that we observed is the physiological basis of this phenotype. What effects could the additional X chromosome have on other cognitive processes or developments that contribute to this observed phenotype? While premature, it is tempting to speculate that the feminization of gene expression that we observed in the BNST/POA is related to partner preference. Although the genes that we found to be feminized have not been previously implicated in the regulation of partner preference, the genetic basis of this behavior remains highly understudied relative to interest in this area, especially from the general public. Due to a variety of constraints, it has proven extremely challenging to study in humans. It has also been difficult to find suitable animal models for this behavior. The best animal model for sexual orientation in human males remains the domestic ram, a species in which ~8% of the population has been found to be exclusively male-oriented [13-15]. Unlike many other animal models, male-oriented rams do not display atypical or feminized sexual behavior. Thus, their partner preference is feminine even though their sexual behavior remains masculine. However, domestic rams are genetically intractable and do not allow for the ease of dissection of genetic pathways that is achievable in mice. As such, we believe that the SCT mice and the experimental paradigm presented in Chapter 2 could prove to be a promising avenue to pursue as it allows the investigation of the role of sex chromosomes on this behavior. To date, the strongest evidence for a genetic basis of male sexual orientation is its association with a region of the X chromosome called Xq28 [16, 17]. Our finding of feminization of partner preference in and expression of both autosomal and X-

linked genes in the BNST/POA of XXY males provides further support for a role of the X chromosome in male sexual orientation. More generally, our findings demonstrate that sex chromosome complement and number can have strong effects on a variety of traits.

Future studies could not only focus on the feminized genes (for instance, by genetically manipulating them and examining if partner preference is affected) but could also investigate the cognitive and behavioral processes that contribute to the partner preference differences seen in the XXY males. We hypothesize that deficits in ultrasonic vocalizations in adulthood could help account for the partner preference phenotype. One of the hallmarks of KS boys is language development deficits. In an analogous manner, ultrasonic vocalizations in XXY male mice may also be deficient in comparison to their XY counterparts. Ultrasonic vocalizations in mice play an important role in mating and reproductive behaviors [18-21]. Male mice emit a characteristic, highly modulated tone that resembles song upon exposure to odors from receptive females and while investigating females prior to the first mounting [22, 23]. It would be of interest to investigate vocalizations emitted by XY and XXY males when presented with (1) a stimulus estrus female and then (2) with a stimulus male. The results from the vocalization studies could help guide future tests of social behavior and partner preference.

The molecular mechanisms of sexual differentiation of the brain

I have established that both hormones and direct genetic effects play important roles in sexually differentiating the brain (Chapters 1 and 2). However, an important question that remains largely unanswered is how cells change on a molecular level in response to these factors. For example, it is known that gene expression patterns are

changed upon exposure to hormones but many of the intermediate steps between initial exposure and the output are still unaccounted for. Perhaps most intriguing is how gonadal hormones and direct genetic effects organize the brain and eventually imprint a male or female identity on undifferentiated neural cells. As a first step to answering this question, we investigated the effect of perinatal testosterone exposure on DNA methylation in the BNST/POA (Chapter 3). We found that exposure to testosterone during the critical period alters methylation patterns at a significant number of genes by day 4 of life (PN4). Unexpectedly, this number was substantially higher in adulthood (PN60). Associated with these changes was a shift in the distribution of methylation sites along the male-female spectrum. At PN 4, DNA methylation in females treated with testosterone was highly feminine, with very few sites showing male-like methylation patterns. By PN60, this distribution had changed dramatically and a large number of sites were now more male-like.

These findings were unexpected. We had hypothesized that testosterone would radically alter methylation patterns of a large number of genes upon perinatal exposure and that this imprint would remain in a stable manner until adulthood (gray dashed line in Fig 4-1). In fact, our data strongly suggest an alternative model where testosterone alters methylation at a relatively small number of genes upon initial exposure. This initial testosterone imprint then becomes amplified during development and ends up affecting a larger number of genes even though testosterone is no longer present. This suggests that organization by testosterone occurs via early programming on relatively few genes and that this small initial effect is what sets up the brain to respond in a particular fashion to other events during postnatal development.

We hypothesize that there are two likely routes for the amplification of the initial testosterone imprint. The first, termed Model A, is represented by the blue line in Fig. 4-1. In this model, DNA methylation changes caused by perinatal testosterone exposure build up gradually over the life of the animal. We think that it is likely that this amplification plateaus at some point in life, most likely adulthood. This gradual buildup is akin to the domino effect: upon perinatal exposure to testosterone, there is a change in DNA methylation in a small number of genes but this limited immediate effect starts a chain reaction which eventually leads to the great differences in DNA methylation patterns seen in adulthood. The second is designated as Model B and represented by the orange line in Fig. 4-1. According to this view perinatal testosterone exposure leads to differential methylation in several dozen genes. This imprint remains stable until a particular developmental period such as the advent of puberty (or adulthood or, in the case of our experimental animals, testosterone pellet implantation at PN45) whereupon there is a second, much larger increase in the number of genes that are affected by testosterone. However, we speculate this second increase only occurs because of the organizational effects of the perinatal testosterone exposure. The cellular and molecular mechanisms responsible for this late-emerging organizational effect remain to be elucidated. The most parsimonious explanation is that this late-emerging effect is due solely to the actions of perinatal testosterone on neural cells. However, an alternative interpretation is that other tissues (for instance, the gonads) are the major site of direct testosterone action and that these tissues then secrete factors that lead to differential methylation in neural cells. We should also consider a potential role for 'master regulator' genes. These would be genes

which are affected shortly after perinatal testosterone exposure and that then go on to affect methylation at many other genes.

As intriguing as these findings are, they represent just a small part of the overall molecular changes that could be occurring within the BNST/POA as a result of testosterone's organizing effects. Testosterone could be affecting other epigenetic mechanisms such as histone modifications, micro RNAs (miRNAs) and 5-Hydroxymethylcytosine (5-hmC). There is compelling evidence implicating all three mechanisms in the processes of sexual differentiation and/or neural development. Levels of histone acetylation in the developing cortex/hippocampus are sexually dimorphic [24]. In addition, regulation of histone acetylation is crucial to sexual differentiation of the principal nucleus of the BNST [25]. A large number of miRNAs show sexually dimorphic expression in the neonatal mouse brain and early prenatal stress can lead to transgenerational dysmasculinization of miRNA expression [26, 27]. 5-hmC is one of the most recently characterized epigenetic marks. Although not much known about its roles, its enrichment in the central nervous system strongly suggest neural cell-specific functions [28]. Furthermore, Szulwach and colleagues found that 5-hmC is regulated in a dynamic fashion throughout postnatal neurodevelopment and aging [29].

Conclusion

What makes someone male or female is a question that has been pondered for millennia. For much of that time, immutable biological differences between the sexes were thought to entirely explain the matter. Centuries of oppression and unequal status were predicated on the idea that women were inherently inferior to men. Unsurprisingly, this led

to a strong backlash against the idea of biological determinism, specifically, and biological explanations for sex differences, generally. The rise of second-wave feminism around the world starting in the 1960s in the United States led to the emergence of a diametrically-opposed viewpoint – men and women were the same in all the ways that mattered and that all the sex differences seen thus far were a result of social conditioning alone. However, the idea that there are biological underpinnings to select sex differences in cognitive ability, career interests and behavior has experienced a resurgence in recent years.

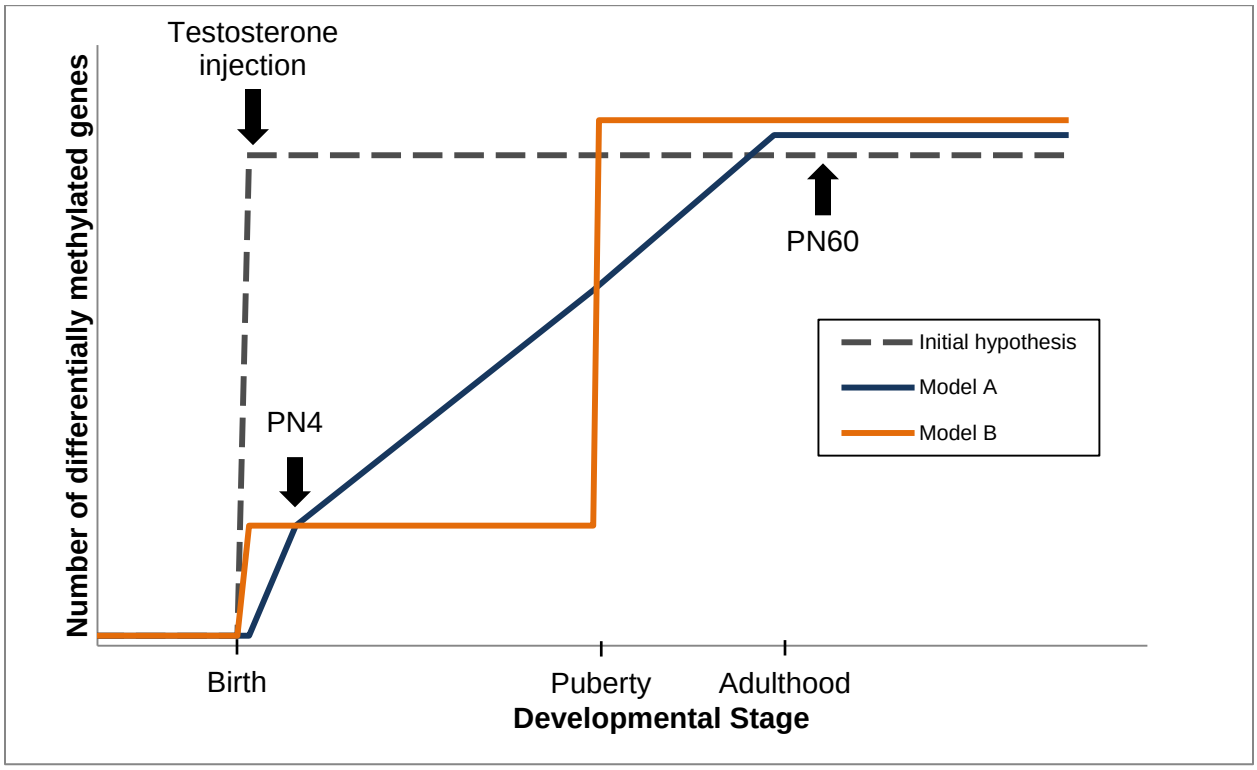
While there has been some fear that the ideas of biological determinism are making a come-back, we must not allow this trepidation to prevent us from doing good science. Medicine used to view women as smaller men and would treat their medical needs accordingly. Due to rigorous research, we now know that approach is fundamentally flawed. By extension, we need to recognize that there are differences between the brains of men and women which are rooted in biology and that these differences may have important implications for how we address issues of neurological and mental health. For example, the pathophysiology of Parkinson's Disease shows sex differences [30]. This suggests that the mechanisms leading to the development of Parkinson's Disease may show differences between men and women and that the most effective treatments and management may need to take the sex of the patient into account. More generally, there is a profound lack of understanding of the neural basis of many sexually dimorphic behaviors and traits such as partner choice and gender identity. Why do the vast majority of women choose men exclusively and vice versa? What are the molecular explanations for this behavior? What are the roles of hormones and genes? The work I have presented in this

dissertation provides us with some clues but there is still a long road ahead of us to fully answer these questions.

The End

FIGURES AND TABLES

Figure 4-1: Model of the late-emerging organizational effect of testosterone on DNA methylation in the BNST/POA. The grey line depicts our initial hypothesis of the fashion in which the organizational effect of testosterone manifests via DNA methylation. The blue and orange lines, Models A and B, respectively, show two likely scenarios of testosterone-mediated organization based on our findings.



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