UCLA
UCLA Electronic Theses and Dissertations

Title
The genetics and epigenetics of sex differences in the brain.

Permalink
https://escholarship.org/uc/item/3jc6p9cc

Author
Ghahramani, Negar

Publication Date
2012

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA

Los Angeles

The genetics and epigenetics of sex differences in the brain

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Human Genetics

By

Negar Montakhab Ghahramani

2012

© Copyright by

Negar Montakhab Ghahramani

2012

ABSTRACT OF THE DISSERTATION

The genetics and epigenetics of sex differences in the brain

By

Negar Montakhab Ghahramani

Doctor of Philosophy in Human Genetics

University of California, Los Angeles, 2012

Professor Eric Vilain, Chair

The major drivers underlying sexually dimorphic brain development are gonadal hormones, namely testosterone (T). During the perinatal sensitive period, a time when the embryonic brain is maximally sensitive to changes in the levels of gonadal hormones, exposure to T has permanent organizing effects on the brain, the molecular basis of which is not known. One potential mechanism for the long term permanence may be DNA methylation. To examine the contribution of epigenetic mechanisms to both the establishment and maintenance of sex differences, I compared the methylomes of male, female, and female mice treated with testosterone. Methylation maps were generated for sexually dimorphic brain regions such as the striatum at postnatal day 4 (PN4) during the sensitive period and PN60 during adulthood using reduced representation bisulfite sequencing. I found that testosterone altered the methylation of a few genes during the sensitive period but a much greater number in adulthood. I next investigated whether administration of a single dose of testosterone to females on the day of birth could induce a shift in DNA methylation from a female-typical to a more male-typical pattern.

The results demonstrated that the masculinizing effect of testosterone was mostly evident at PN60 but not at PN4. This observation provided a new perspective on the mechanisms underlying organizational effects of testosterone because contrary to the expectation that testosterone leaves a strong, stable imprint shortly after exposure, testosterone effects on DNA methylation were not immediately evident but emerged later. Based on these data, I concluded that sex differences in methylation are not the result of the immediate early actions of testosterone on the brain. Rather, the neural molecular patterns found in adults are conditioned by early hormonal exposures, the effects of which might emerge over a period of time. Gene Ontology analysis on the set of genes whose methylation was altered by testosterone revealed a significant enrichment of genes belonging to signaling components associated with dopamine modulation as well as movement disorders that display a male-bias. These data are consistent with striatum's role in regulation of movement.

In addition to assessing the contribution of hormones to brain sexual differentiation, I also investigated the impact of sex chromosomes on sex differences in brain and behavior. To test for sex chromosome effects, I used the four core genotypes mouse model and found sex differences in expression of a subset of striatal genes caused by XX vs. XY differences in mice with the same gonadal type. Moreover, comparison of animals with different numbers of sex chromosomes in a novel mouse model of Klinefelter Syndrome (KS), the Sex Chromosome Trisomy Model, indicated that presence of an additional X chromosome and/or its interaction with the Y in XXY male mice can contribute to some of the behavioral and molecular phenotypes observed in KS. Interestingly, analysis of striatal transcriptome in KS mice revealed a feminized molecular signature in the brain of KS male mice. Such information is crucial

knowledge in elucidating not only the pathophysiology of KS, but also the origin of sex differences in brain and behavior.

Altogether, my work demonstrates the significance of genetics and epigenetics in the process of brain development as it relates to sex. The results presented in this dissertation suggest that (1) the sex chromosomes carry genes that could influence brain function and behavior; and (2) the long lasting effects of steroid hormones on the brain could be mediated by epigenetic mechanisms such as DNA methylation.

The dissertation of Negar Montakhab Ghahramani is approved.

Arthur P. Arnold

Stephen Cederbaum

Karen Reue

Eric Vilain, Committee Chair

University of California, Los Angeles

2012

DEDICATION

I dedicate this dissertation to my family, especially…

to Kambiz, the love of my life;

to Dad and Mom, Abdi and Firouzeh, for instilling the importance of hard work

and higher education;

to late Fatima, who taught me unconditional love;

to late grandma, late grandpa, Abbas and Azam Firouzi, whose memories I will cherish in my heart forever;

to my American family, Dr. Massoum Montakhab, and Dr. Nikou Hessami—for giving me the opportunity to come to United States and their love and support all along.

I love you all dearly.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

ACKNOWLEDGEMENTS

Foremost, I would like to express my sincere gratitude to my advisor Prof. Eric Vilain for the continuous support of my Ph.D. study and research, for his patience, motivation, enthusiasm, and immense knowledge. He is one of the most wonderful and smartest scientists I know. His guidance has helped me in all the time of research and writing of this thesis. I could not have imagined having a better advisor and mentor for my Ph.D. study.

I cannot find words to express my gratitude to my co-mentor, Dr. Arthur Arnold, for his detailed and constructive comments, and for his important support throughout my research. I consider it an honor to work with him. His ideals and concepts have had a remarkable influence on my entire career in the field of sex differences research.

I share the credit of my work with my colleague and very dear friend, Tuck Ngun, for whom I have great regard. I thank him for his support all throughout my work, the stimulating discussions, and for all the fun we have had in the last five years. His friendship has been of great value in my studies.

I would also like to express my gratitude to Drs. Nancy Forger and Geert de Vries for providing constant support and encouragement as well as for the fruitful discussions and expert advice that enabled me to complete my dissertation. During this work I have collaborated with many other professors and colleagues (Dr. Matteo Pellegrini, Dr. Pao-Yang Chen, Dr. Yuichiro Itoh, and Dr. Janet Sinsheimer) and I wish to extend my warmest thanks to them for their valuable advice and friendly help particularly in the area of bioinformatics and statistical analysis that have been instrumental in my PhD study. I would like to thank all past and present Vilain lab members: Dr. Alice Fleming, Dr. Ruth Baxter, Dr. Francisco Sanchez, and Dr. Valerie Arboleda for all of their help. It is a pleasure to work in a dynamic team of researchers who

provide me with valuable experiences both within and outside of work. Finally, I would also like to sincerely thank my committee members, Dr Karen Reue and Dr Stephen Cederbaum, for being an active part of my committee as well as providing me with insightful advice on my work.

I would like to make a special reference to the love of my life, my husband, Dr. Kambiz Aghili. Without his encouragement and understanding it would have been impossible for me to finish this work. He has always stood by me and believed in me. I owe my loving thanks to my parents, Firouzeh Firouzi and Abdi Ghahremani, and to my American family, Dr. Montakhab and Dr. Nikou Hessami for teaching me the value of education. I am who I am today because of their sacrifices and inspiration. Special thanks to my brother, Nami Ghahramani, who has always believed that I could do it.

Lastly, I would like to clarify that I was responsible for the experimental design, and analysis of the data obtained from the striatum. Tuck Ngun primarily contributed to the work done on the bed nucleus of the stria terminalis/preoptic area (BNST/POA).

Chapter 1 contains excerpts from a review with the following citation: Ngun, T. C.*, Ghahramani, N.*, Sanchez, F. J., Bocklandt, S., Vilain, E. The genetics of sex differences in brain and behavior. Front Neuroendocrinol. 2011. **32**(2): p. 227-46. Review. * denotes equal contribution.

Chapter 2 contains data for future publication. Author list: Negar M. Ghahramani, Yuichiro Itoh, Xuqi Chen, Stephanie Muir, Sangitha Krishnan, Rebecca McClusky, Arthur P. Arnold, and Eric J. Vilain. Dr. Xuqi Chen helped with the dissections. Sangitha Krishnan and Stephanie Muir helped with the methylation experiments and analysis of the data. Dr. Yuichiro Itoh helped with the statistical analysis. Dr. Arthur Arnold and Dr. Eric Vilain were joint PIs on this project.

Chapter 3 includes data for future publication. Author list: Negar M. Ghahramani*, Tuck C. Ngun*, Shayna Williams, Michelle N. Creek, Hayk Barseghyan, Yuichiro Itoh, Francisco J. Sánchez, Rebecca McClusky, Janet S. Sinsheimer, Arthur P. Arnold, and Eric Vilain. * denotes equal contribution. Tuck Ngun helped design and execute the experiments and performed analysis of the gene expression data from the BNST/POA. Shayna Williams designed, executed and analyzed the motor behavior tests. Hayk Barseghyan aided with the analysis of the partner preference tests. Michelle Creek, Dr. Janet Sinsheimer, Dr. Francisco Sanchez, and Dr. Yuichiro Itoh helped with the statistical analyses. Dr. Arthur Arnold and Dr. Eric Vilain were joint PIs on this project.

Chapter 4 includes data for future publication. Author list: Negar M. Ghahramani*, Tuck C. Ngun*, Pao-Yang Chen, Tara TeSlaa, Sangitha Krishnan, Stephanie Muir, Matteo Pellegrini, Arthur P. Arnold, Geert J. de Vries, Nancy G. Forger, and Eric Vilain. * denotes equal contribution. Tuck Ngun helped design and execute the experiments and performed analysis of the methylation data from the BNST/POA. Dr. Pao-Yang Chen and Tara TeSlaa performed the bioinformatic and statistical analyses. Sangitha Krishnan and Stephanie Muir helped with data analysis from both the BNST/POA and the striatum. Dr. Matteo Pelligrini, Dr. Arthur Arnold, Dr. Geert de Vries and Dr. Nancy Forger helped with the planning, design and execution of the experiments. Dr. Eric Vilain was the PI of this project.

NEGAR GHAHRAMANI

ACADEMIC BACKGROUND

- **Master of Science** (GPA 3.91), Molecular Biology, San Diego State University [2006]
  Thesis: Heat shock proteins as regulators of autoimmune inflammation in Crohn's disease
  + Certificate in Biotechnology [2003]
- **Bachelor of Science** (Honors), Cellular & Molecular Biology, Tehran University [2002]

RESEARCH EXPERIENCE

- **UCSD, Dept. of Pediatrics** ***Autoimmune Diseases*** [Research Associate, 2006 – 2007]
  - Characterization of the role of immune deviation in rheumatoid arthritis.
- **Androclus Therapeutics**, San Diego ***Autoimmune Diseases*** [Research Associate, 2002 – 2006]
  - Implementation/study conduct and reporting results of multiple sclerosis, rheumatoid arthritis, and Crohn's disease pre-clinical animal studies.
- **Molecular Diagnostic Services**, San Diego [Summer Research Assistant, 2000, 2001, 2002]
  - Acquired substantial knowledge and hands on experience in cellular and molecular biology and cytogenetic techniques (e.g., FISH analysis)

AWARDS

- **Leena Peltonen Award in Research Excellence at UCLA** [November 2012]

PUBLICATIONS

- **N. Ghahramani***, T. C. Ngun*, P. Y. Chen, M. Pellegrini, A. P. Arnold, G. J. de Vries, N. G. Forger, and E. Vilain. (2012). Epigenetic modifications during brain sexual differentiation. (*Manuscript in preparation*) - (* *Equal contribution*)
- **N. Ghahramani***, T. C. Ngun*, H. Barseghyan, Y. Itoh, A. P. Arnold, and E. Vilain. (2012). Brain and behavior phenotypes in an animal model of sex chromosome disorders. (*Manuscript in preparation*) - (* *Equal contribution*)
- **N. Ghahramani***, R. Billetta*, O. Morrow, B. Prakken, H. D. Jong, C. Meschter, P. Lanza, and S. Albani. (2012). Epitope-specific immune tolerization ameliorates experimental autoimmune encephalomyelitis. Clin Immunol. 145(2), 94-101 - (* *Equal contribution*)
- A. Fleming, **N. Ghahramani**, M. X. Zhu, E. C. Délot, and E. Vilain. (2012). Membrane β–catenin and adherens junctions in early gonadal patterning. Dev Dyn. 241(11), 1782-98.
- **N. Ghahramani***, T. C. Ngun*, F. J. Sánchez, S. Bocklandt, and E. Vilain. (2011). The genetics of sex differences in brain and behavior. Front Neuroendocrinol. *32*(2), 227-46. (* *Equal contribution*)
- S. T.A. Roord, E. Zonneveld-Huijssoon, T. Le, G. Puga Yung, E. Koffeman, A. Ronaghy, **N. Ghahramani**, P. Lanza, R. Billetta, B. J. Prakken, and S. Albani. (2006). Modulation of T cell function by combination of epitope specific and low dose anticytokine therapy controls autoimmune arthritis. PLoS One. *1*(1), e87.

PRESENTATIONS ……..……...

- Ghahramani N.M. (2012). Genetic and Epigenetic Influences on Brain Sex differences. Laboratory of Neuroendocrinology/Training Program in Neural Repair Joint Seminar Series, University of California, Los Angeles.
- Ghahramani N.M. (2011). Genetic Influences on Brain Sex differences. UCLA Human Genetics Retreat, University of California, Los Angeles.
- Ghahramani N.M. (2011). The Genetics and Epigenetics of Hormonal Influences on Brain Sexual Differentiation. Laboratory of Neuroendocrinology, University of California, Los Angeles.
- Ghahramani N.M. (2010). Genetics and Epigenetics of Sex Differences in the Brain. Laboratory of Neuroendocrinology, University of California, Los Angeles.
- Ghahramani N.M. (2009). Exploring the Mysterious Role of *Sry* in the Brain. Laboratory of Neuroendocrinology, University of California, Los Angeles.

POSTERS ……..……...

- **Ghahramani N.M.***, Ngun T.C. *, Chen P.Y., Muir S., Krishnan S., TeSlaa T., Pellegrini M., Arnold A.P., de Vries G.J., Forger N.G., and Vilain E. (April 2012). The Epigenetic underpinnings of brain sexual differentiation. 6th International Symposium on Vertebrate Sex Determination, Kona, Hawaii. (* *Equal contribution*)
- Ngun T.C.*, **Ghahramani N.M.***, Barseghyan H., Creek M., Itoh Y., Sánchez F.J., McClusky R., Williams S., Sinsheimer J., Arnold A.P, and Vilain E. (April 2012). Partner preference in a mouse model of sex chromosome trisomies. 6th International Symposium on Vertebrate Sex Determination, Kona, Hawaii. (* *Equal contribution*)
- **Ghahramani N.M.***, Ngun T.C. *, Chen P.Y., Muir S., Krishnan S., TeSlaa T., Pellegrini M., Arnold A.P., de Vries G.J., Forger N.G., and Vilain E. (June 2012). Epigenetic modifications during brain sexual differentiation. Joint Meeting of OSSD & IGM, Baltimore, MD. **SECOND-PRIZE WINNER.** (* *Equal contribution*)
- Ngun T.C.*, **Ghahramani N.M.***, Barseghyan H., Creek M., Itoh Y., Sánchez F.J., McClusky R., Williams S., Sinsheimer J., Arnold A.P, and Vilain E. (June 2012). Brain and behavior phenotypes in a mouse model of sex chromosome trisomies. Joint Meeting of OSSD & IGM, Baltimore, MD. (* *Equal contribution*)
- **Ghahramani N.M.***, Ngun T.C. *, Chen P.Y., Muir S., Krishnan S., TeSlaa T., Pellegrini M., Arnold A.P., de Vries G.J., Forger N.G., and Vilain E. (October 2012). The epigenetic underpinnings of brain sexual differentiation. Neuroscience 2012, SfN's 42nd annual meeting, New Orleans, LA. (* *Equal contribution*)
- Ngun T.C.*, **Ghahramani N.M.***, Creek M., Williams S., Barseghyan H., Sánchez F.J., Itoh Y., McClusky R., Sinsheimer J., Arnold A.P, and Vilain E. (October 2012). Feminization of partner preference and brain gene expression in XXY males from the Sex Chromosome Trisomy model, a novel mouse model of Klinefelter Syndrome. Neuroscience 2012, SfN's 42nd annual meeting, New Orleans, LA. (* *Equal contribution*)

PROFESSIONAL AFFILIATIONS ……...

- American Society of Human Genetics
- The Organization for the Study of Sex Differences (OSSD)
- Society for Neuroscience (SFN)

Chapter 1

Introduction

OVERVIEW

The brains of men and women demonstrate differences in various aspects that range from morphology to chemistry to function [1]. Most studies conducted so far have focused on morphological brain differences—namely, the size or volume of specific regions or nuclei. Yet, it is now known that the two sexes exhibit differences that are not only related to differences in neural gross morphology, but also to many finer level processes including neuronal density [2-4], synaptic adaptation, patterns of brain activation [5, 6], and neurochemistry [7-10].

Men and women also differ in many psychological and behavioral aspects (Table 1-1). For example, men perform better on specific visuospatial aspects (e.g., mental rotation) compared to women; and women perform better on specific verbal tasks (e.g., verbal fluency) compared to men [11]. There is also a large sex difference in sexual interests and behaviors [12, 13].

Some contend that these differences are due to social systems and gender socialization [cf. 14, 15, 16]. Nevertheless, many of the reported sexual dimorphisms have been attributed to various biological factors. Thus, a more thorough understanding of the main biological determinants involved in expression of sex differences can help us better explain the relationship between brain, behavior, and environment. In addition, it could allow us to determine how one's sex influences the risk of developing disorders that manifest and progress differently in men and women (e.g., autism, schizophrenia and Parkinson's disease [17-21]) (Table 1-2). Such knowledge can better inform the treatment of these diseases.

In fact, great advances have been made in the field of brain sex differences, underscoring the role of sex steroid hormones such as estrogen and testosterone in producing sexually dimorphic changes in brain morphology that could ultimately lead to behavioral differences.

Secretion of testosterone from the gonads and its aromatization to estradiol in the brain (during a perinatal time window in which the brain is highly responsive to gonadal secretions) causes permanent and sexually differentiated organizational changes that could ultimately shape the male and female sexual and other behaviors during adulthood [22]. Manipulation of hormone levels in animal models impedes normal sexual differentiation of many sexually dimorphic phenotypes, which leads to the dogma that gonadal hormones are the main causative agents of sexual differentiation [23, 24]. Thus, it is not surprising that a great deal of effort has been made in understanding the cellular and molecular mechanisms underlying the effects of gonadal secretions.

Although most of these studies have focused on the organizational effects of gonadal hormone levels (those permanent effects that act during the fetal/neonatal period to set up the capacity to show behaviors typical of females or males during adulthood), this does not exclude the presence of activational effects. In fact, the differences that are caused by activational hormone effects (those transient effects of steroids most typically associated with adulthood that facilitate behaviors codified by early exposure to gonadal hormones) exemplify the importance of hormone-driven mechanisms in producing such sex differences and suggest that there is a complex cross talk between early organizational effects and adult brain physiology and behavior [22, 25].

Moreover, in order to develop a more comprehensive perspective on how the brain sexual differentiation occurs, other variables (including the contribution of genetic and epigenetic effects) should also be taken into account [26-29]. Recently, it was found that gonadal hormones are not the only determinants of sexual dimorphism, but rather other factors could also contribute

to brain sex differences. Sex chromosomes carry genes that can influence sex-specific differences observed in neurodevelopment, brain function, and behavior of mammals prior to gonadal differentiation. Therefore, studying the effect of X- and Y-linked genes, termed direct genetic effects, may further provide interesting clues about the biological basis of sex differences in the brain.

Epigenetic modifications are also implicated in brain sexual differentiation and provide a plausible mechanism by which early and permanent gonadal hormones can mediate their effects on gene expression differences. These factors, especially DNA methylation, can modulate the effects of hormones on the developing brain [30, 31].

Based on these observations, we first outline the past and current theories of molecular mechanisms of brain sexual differentiation (Chapter 1). We discuss the relative contribution of hormones to brain sexual differentiation. We then present recent evidence that not all sex differences are controlled by differences in the levels of gonadal hormones and that the direct effects of genes encoded on the sex chromosomes can also contribute to sexually differentiated phenotypes (Chapters 2 and 3). We explore some of the primary approaches for studying sex-specific variables that cause sexual differentiation. Finally, we review the present data on how epigenetic changes in the nervous system are emerging as a critical component of long-lasting effects induced by early life hormonal exposure (Chapter 4).

Early Hormonal Theories of Sexual Differentiation

The term “sexual differentiation” refers to a series of events (sex determination and sex differentiation) in which the sexually undifferentiated fetus progressively develops male- or female-typical characteritics. Sex determination, which depends on the genetic sex of the embryo,

drives the development of either a testis, in the presence of the Y chromosome, or an ovary in the absence of it and the presence of a second X chromosome. Sex determination sets the stage for sex-specific responses of tissues to gonadal secretions which is referred to as sex differentiation. The cellular and morphological events that occur during early sexual development and the underlying molecular events that underpin this process has been the subject of many research studies in the past.

Frank Lillie was among the first to recognize the value of the sex steroid hormones in the process of sexual differentiation. He proposed that freemartins were indeed zygotic females partially masculinized by the sex steroid hormones produced by their male co-twin during gestation. According to his assessments, a hormone secreted from the fetal testes of the zygotic male twin passes through the fused extraembryonic circulations, reaches the zygotic female twin, and masculinizes the development of gonads. His analysis of freemartins, therefore, suggests that the masculine development of the internal reproductive structures in the freemartins were mainly determined by the hormonal milieu of the fetal environment [32].

The hormonal theory of sexual differentiation was further strengthened by experiments carried out by Alfred Jost. He showed that upon surgical castration of fetal rabbits, development of the internal duct systems (the external genitalia and the Wolffian duct system that normally regresses in females but in males develops into the epididymis and vas deferens) resembles a female pattern. The external genitalia proceeded to be female-like. The Mullerian duct (which normally disappears in males, but in females leads to development of the uterine tubes, the uterus, and part of the vagina) persisted in both male and female experimental animals. Jost also injected testosterone into female fetuses and demonstrated that there was a male-typical development of both external genitalia and the Wolffian duct. From these observations, Jost proposed the idea that the testis produces hormones that contribute to the masculine development

of internal and external male structures, and suppression of the development of the female organs [33, 34].

Organizational Hormone Effects in the Brain

The abovementioned studies formed the theoretical foundation for a series of experimental work in following years, which demonstrated that genetic sex (the complement of sex chromosomes) defines gonadal sex (the sex determination process) and that steroid hormones secreted by the gonads acted sex-specifically to determine the development of other internal structures besides the gonad, such as the brain (the process of sex differentiation).

However, Phoenix, Goy, Gerall and Young in their 1959 publication in the journal *Endocrinology* were among the first line of researchers to show that early hormone exposure during development permanently organizes behavior in adulthood [24]. They administered testosterone propionate to pregnant guinea pigs and demonstrated that the mating behavior of the female offspring was masculinized during adulthood under permissive conditions (gonadectomy before puberty and injection with estradiol benzoate and progesterone for lordosis induction or testosterone propionate injection for masculine mounting). This study showed that testosterone secreted by testes during the critical period has a permanent masculinizing effect on the brain. It has the potential to act on brain regions that control mating behavior, as well as the ability to organize the responsiveness to gonadal hormones that activate behaviors in adulthood.

Since this seminal publication, many studies have looked at the physiological impact of hormones on the developing brain. These studies demonstrate that hormones such as testosterone and estrogen are critical to masculinization and feminization of brain areas that are critical to the normal expression of adult sex behavior. For instance, in rodents, testosterone is converted

locally in the brain to estrogen via the enzyme p450 aromatase during the perinatal sensitive period, and disrupting the function of the aromatase-encoding gene impairs masculine brain development [35]. Besides aromatase, loss of function of estrogen receptor alpha and estrogen receptor beta in double knockout mice results in complete abolishment of male sex behavior [36-38], suggesting that sex differentiation of the brain is a process driven by gonadal hormones. These data, in conjunction with the findings that implicate androgen and androgen receptor in adult sex behavior [39], have led to the dogma that gonadal hormones are the main causative agents of sexual differentiation [23, 24].

Even today, gonadal steroid hormones are playing an increasingly important role in the process of brain sexual differentiation and the vast majority of neural sex differences can be explained by organizational hormone effects. Sexual dimorphisms in many sites and subnuclei of the brain have been shown to be estrogen- or androgen-driven. For example, the medial preoptic area (MPOA), one of the most sexually dimorphic areas of the brain involved in regulation of male sex behavior and control of gonadotropin release, contains several subnuclei (SDN-POA and AVPV) that display sex differences in volume. The basis for these differences stems from estrogen-regulated alterations in cell survival and cell death (while estrogen prevents a BAX/ caspase 3 associated cell death in the male neurons of the SDN-POA [40]; it induces caspase-dependent cell death and promotes TNF-a-NF-kB mediated cell survival in the male dopaminergic [41] and female GABAergic neurons [42] of the AVPV, respectively).

Steroid hormone-dependent differential cell death and/or survival are not the only major factors contributing to sex differences in the brain; dendritic plasticity could also evoke brain sexual differentiation. For instance, in the male preoptic area prostaglandin synthesis in the brain

enhances PKA-induced dendritic spine density and results in maintenance of AMPA/kainite glutamate receptor expression on the dendritic spines [43-45]. Interestingly, interference with any of the steps along this pathway has been shown to cause sex reversal in rodents during the critical period. Another example of an estradiol-affected dendritic spine formation has been observed in the ventromedial nucleus (VMN) of the hypothalamus which is the main region involved in controlling female sex behavior [46]. Interestingly, VMN neurons in the male contain more dendritic spines and therefore more elicit more excitation compared to females. Construction of dendritic spines in this brain region is estrogen receptor mediated [47, 48]. Estrogen receptor activation leads to enhanced pre-synaptic glutamate release (phosphoinositide 3-kinase dependent) and an increase in the activity of post-synaptic NMDA receptors [49].

Testosterone also plays a significant role in the process of brain sexual differentiation. For instance, the SNB nucleus (the spinal cord nucleus that controls striated muscles of the penis) is larger and contains more cells in male mammals than in females [50]. Testosterone plays a key role in this process since the sex difference in cell number can be fully reverted by treating females neonatally with androgen [51] or disrupting the function of the androgen receptor in male rats, implicating a role for neonatal androgen state during the critical period in SNB masculinization [52].

The bed nucleus of the stria terminalis (BNST) is another brain region influenced by testosterone during the critical period. This limbic forebrain structure is involved in the regulation of sexual behavior, gonadotrophic release, stress and anxiety. The principal nucleus of the BNST (BNSTp) is larger in volume in males than in females. In rats and mice, the sex difference in volume is due to the presence of twice as many dying cells in the BNSTp in the

females during postnatal life. Testosterone completely masculinizes BNSTp cell number through a bax-dependent cell death pathway [53].

Organizational effects of testosterone also play an important role in the sexual differentiation of other brain areas such as the corpus callosum. Male rats have larger callosal area, length, and perimeter compared to female rats. Testosterone given neonatally to females clearly enlarged their callosa, indicating a role for this sex steroid hormone in masculinization of this brain region [54]. Altogether, these observations point to sex-specific organizing roles for estrogen and testosterone in modulating neurotransmission, neuronal morphology, cell survival, and cell death.

It is also recognized that puberty may be another sensitive period for steroid-dependent brain sexual differentiation and that these pubertal hormones have the ability to further organize the neural circuits that mediate adult behavior. The organizational effects of gonadal hormones during puberty build on the action of hormones secreted by the gonads during the perinatal sensitive period and appear to have permanent consequences on brain structure and function across a wide variety of species, including humans [55-57].

Exceptions to the Classical View

The traditional view that the hormones are the only determinants of brain sexual differentiation is shifting. Currently, there is ample evidence to support the idea that factors other than the hormonal milieu, including genetic and epigenetic, act in concert to bring about brain sex differences. Underlying mechanisms have shown to be diverse and brain region-specific and not restricted to areas concerned with reproductive physiology and behavior. Genes that are expressed from the X or Y chromosomes could account for some of the sex differences observed

in non-gonadal cells. Epigenetic mechanisms could also act as potential "mediators between the sex and the genetic blueprint" by fine-tuning brain gene expression [58].

Several of the findings that question the role of gonadal hormones as the sole determinants of brain sexual differentiation are presented below: Research performed on the zebra finch was among the first to suggest a role for factors other than gonadal hormones. In zebra finches, males exhibit courtship behaviors that are unique to their sex; specifically, the potential to sing a distinct courtship song. This ability has been attributed to several brain regions that are larger in males compared to females [59, 60]. Given the hypothesis that sex-specific hormones are the only determinants of such differences, several researchers unsuccessfully attempted to alter the courtship behavior of finches by just manipulating hormone levels [61]. It was shown that blocking hormone action in males does not fully prevent masculine development [62]. Furthermore, female zebra finches that developed testes continued to develop feminine song circuitry and did not exhibit masculine song behavior [63, 64]. In addition, Jacobs et al. treated female zebra finches with estrogen at the beginning of hatching [65]. Interestingly, estrogen treatment was not able to cause full masculinization of the neural circuitry of the zebra finch song system; the song circuitry was still smaller compared to control males [66, 67], and supraphysiological doses of estrogen were required for full masculinization [68]. Similarly, it was shown that inhibiting the action of estrogen by using aromatase blockers in males does not completely prevent the male differentiation pathway [59, 69-72].

The discovery of a rare type of zebra finch provided support for the hypothesis that sex chromosome genes might play a role in sexual differentiation. The lateral gynandromorphic finch has male-typical phenotypes on one half of the body (e.g., plumage, testis, and song

circuitry) and female-typical phenotypes on the other half of the body. Each half of such finches is either entirely genetically male or genetically female. Thus, each side contains the sex-specific genes necessary for the development of the corresponding sex-specific traits. In this model, while the gonadal hormonal actions in producing sex differences in the brain cannot be completely ruled out (both sides of the neural song system were larger than that of normal females), their influences cannot fully explain the differences observed between the left and right sides of the brain. Given this explanation, the most reasonable theory is that endogenous genetic differences in the brain cells themselves can also contribute to the unequal differentiation of the two sides producing sex differences through their local action within the brain [73].

The idea that perhaps the genetic sex of the brain cells can also result in sexual differentiation of the brain, was further strengthened by a recent study on gynandromorphic chickens. Zhao et al. demonstrated that, the 'sex identity' of somatic cells in the birds in not driven by the gonadal hormone milieu, and the sex chromosome complement of these cells (not their hormonal environment) leads to expression of sex-typical phenotypes in these cells [29, 73]. In contrast to mammals where XX somatic cells can turn into Sertolli cells and XY cells can develop into granulosa cells when introduced into the sex-atypical gonads [74, 75], in chickens, XY cells that had been transplanted into an ovary continue to express male-specific markers. Since the gonadal hormonal environment is the same for both the donor and host somatic cells, the sex chromosome complement makes them respond differently.

A second exception to the classical view that we highlight below concerns the development of the tammar wallaby, a marsupial that is much smaller than the kangaroo. As with brain development, gonadal hormones drive the sex-specific development of the external

genitalia in most mammals. Specifically, androgens promote the development of male genitalia. However, the formation of reproductive structures in the tammar wallaby appears to be independent of gonadal hormone control and is solely due to the effect of sex chromosome complement.

During the fetal development of the tammar wallaby, the production of testosterone, which would typically masculinize mammalian fetuses, does not occur in these marsupials until about the fourth or fifth day after birth [76-79]. Yet, signs of sex-specific reproductive structures (e.g., scrotum, mammary gland, and pouch formation) can be observed as early as several days before birth. In mammals, the development of male-specific structures is thought to be completely dependent on the action of androgens [80]. Experiments that increased or decreased the action of testosterone or estrogen in the tammar wallaby had no significant effect on the development of the external genitalia [81, 82]. This suggested that such differences were not under gonadal hormone control.

A case similar to the gynandromorphic zebra finch has also been reported in tammar wallabies: This consists of wallabies that are XX on one side of the body and XY on the other side of the body. Such wallabies develop a hemipouch on the XX side and a hemiscrotum on the XY side even after exposure to circulating gonadal hormones [68, 83, 84]. As with the zebra finch, such cases challenged the view that all sex differences were due to hormones produced by the gonads.

Brain Sex Differences and Direct Genetic Effects

Sex differences in the brain may contribute to some of the psychological and behavioral differences we observe between the sexes. Furthermore, they may influence the susceptibility to

different diseases. For instance, Parkinson's disease—a neurodegenerative disease that impairs motor function and speech—affects more men than women. Research has established a link between Parkinson's disease and a loss of dopaminergic neurons in the substantia nigra [85]. Such losses disrupt dopamine pathways, which leads to many of the symptoms associated with Parkinson's disease.

Robust sex differences have been observed in the development, activity, and number of dopaminergic neurons. The data described below represents a clear example of a sex difference in the brain that has a strong genetic component.

A. Dopaminergic neurons 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 [86]. Furthermore, females had higher numbers of dopaminergic, tyrosine hydroxylase-immunoreactive (TH-ir) cells in the midbrain, and their mesencephalic and diencephalic neurons produced more dopamine when compared to males. On the other hand, soma measurements of diencephalic neurons from male cultures contained larger dopaminergic neurons. Although it is difficult to make accurate measurements of hormonal levels in the embryonic brain, it is unlikely that there is a huge sex difference due to gonadal hormone exposure at this stage as the rat gonad only *begins* to differentiate at this point. Therefore, this suggests a contribution of sex chromosome complement and/or sex-specific gene expression.

These differences in dopamine neurons are not altered even when gonadal hormone levels are manipulated. Specifically, treatment with estradiol and testosterone does not eliminate the observed sex differences in number, size, or function of the dopaminergic cells. Similar findings were later replicated in a study using mesencephalic cultures from the NMRI strain of mice [87]. Collectively, these observations strongly support the idea that some of the sex-specific properties of the dopaminergic neurons appear to be under the control of non-hormonal mechanisms.

B. The *Sry*'s role in dopaminergic neuron development

Further studies have convincingly supported the idea that direct genetic effects play an important role in neural sex differences. For instance, it has been recently shown that the Y-encoded testis-determining gene, *Sry*, directly functions in the brain to influence neural development and sex-specific behaviors. In humans, *Sry* mRNA is expressed in tissues outside of the gonad including brain regions such as the hypothalamus, frontal, and temporal cortex [88]. It co-localizes with tyrosine hydroxylase (TH)-positive neurons of the substantia nigra pars compacta (SNc) and ventral tegmental area (VTA) or GABAergic (GAD67-positive) neurons in the human male substantia nigra pars reticulata (SNr) [89]. In the human male dopaminergic cell line M17, downregulation of *SRY* reduces the expression levels of *TH*, monoamineoxidase-A (*MAO-A*), dopa decarboxylase (*DDC*) and dopamine β hydroxylase (*DBH*). *SRY* over-expression on the other hand leads to an increase in *TH*, *MAO-A*, *DDC* and *DBH* mRNA levels concomitant with a modest but significant increase in dopamine levels [89]. When human teratocarcinoma NT2 cells, which express *SRY*, are differentiated with retinoic acid into NT2N dopamine neurons, the differentiated cells begin to express elevated levels of SRY in conjunction with dopamine neuron markers [e.g., TH, nuclear receptor related 1 (NURR1) and dopamine receptor

D2 (D2R)] [90, 91]. In fact, Czech et al.'s work demonstrates that SRY activates a human 4.6 kb fragment of the *TH* promoter, a region involved in *TH* regulation in the SNc of transgenic mice [89].

Sry mRNA is also found in the midbrain and hypothalamus of adult male mice in several developmental stages [92] and appears to control tyrosine hydroxylase (TH) expression particularly in the brain and adrenal medulla [93].

Milsted et al. showed that SRY and TH are both present in the locus coeruleus, substantia nigra, and ventral tegmental area of the male rat and that SRY upregulates TH expression *in vitro* [93].

Importantly, an experiment carried out by our own group demonstrated that SRY is expressed and co-localized with TH in the substantia nigra, medial mammillary bodies of the hypothalamus, and the cortex of male but not female rats [94]. Furthermore, when SRY is downregulated, specifically in male rats in the tyrosine hydroxylase-expressing neurons of the substantia nigra (SN) by antisense oligonucleotides, it decreases nigrostriatal tyrosine hydroxylase expression, and results in marked depletions of striatal dopamine levels and deficits in motor performance.

A regulatory role for SRY on dopamine was further confirmed in other studies that showed that SRY acts via TH in rodents, MAO-A in rodent neuronal N2a cells and human BE(2)-C cell line to regulated dopamine [95-99]. Altogether, these data suggest a direct sex-specific effect of a Y-linked gene in establishing sex differences in neural function and behavior [94].

Sex differences in the striatum

In this section, we briefly review the biological bases of sex differences in the striatum since almost all our experiments were done on this brain region and we suggest that gonadal hormones contribute to some, but not all of these differences.

The striatum, which is the main region involved in dopaminergic function and reward, displays several key sex differences, many of which are caused by gonadal steroid hormones such as estrogen. Indeed, it has been shown that estrogen exerts both activating and inhibiting effects on the dopamine system.

In rats, dopamine biosynthesis [100], concentration [101, 102], degradation [103], uptake [104, 105], and receptor density [106] vary during the estrous cycle. In female but not male rats, there is an estrous cycle-dependent variation of: (1) basal extracellular concentration of striatal DA [107]; (2) amphetamine (AMPH)-stimulated DA release [107]; and (3) striatal DA-mediated behaviors [107]. During estrous, AMPH- or cocaine-induced behaviors are elevated compared to other days during the cycle. Ovariectomy lessens and weakens basal extracellular DA, AMPH-induced striatal DA release, and behaviors mediated by the striatal DA system. Estrogen also acts directly on the striatum and accumbens via a G-protein–coupled external membrane receptor to enhance DA release and DA-mediated behaviors [107].

Several studies have also highlighted the association between estrogen levels and dopamine transmission in humans. However, inhibiting effects of estrogen are mostly reported. Women, for example, have potentially lower D2 receptor affinity than men, and this difference is particularly observed in the left striatum and has been attributed to estrogen levels. Women's lower D2 receptor affinity is suggestive of increased endogenous striatal dopamine

concentration, which may help explain men and women's differing susceptibility to psychiatric disorders [108].

As mentioned above, however, there have also been observed sex differences in striatal DA transmission that appear to be caused independently of hormones. Recent studies have shown that SRY is specifically expressed in the dopaminergic neurons of the substantia nigra in adult male rodents where it enhances striatum DA release and regulates sensorimotor functions of dopaminergic neurons [95]. Such data adds onto the increasing evidence that along with the effect of hormones, sex chromosome-linked genes are critical to sexually dimorphic neural and behavioral traits.

Strategies to study sex differences in the brain

In light of the scientific findings mentioned above, the field of sexual differentiation now commonly examines the genetic bases of sex differences alongside gonadal hormone effects. Contrasting the effects of genotype from gonadal hormones has not been easy since the two variables almost always go hand in hand.

In the following sections, we highlight the 'four core genotypes' and the 'Sex Chromosome Trisomy' mouse models, which have proven to be powerful tools in teasing out the effects of gonadal versus chromosomal sex.

A. <u>The 'Four Core Genotypes' Model</u>

A 2x2 mouse-model was developed to separate the effects of gonadal sex from chromosomal sex. This model, known as the 'four core genotypes' (FCG), allows researchers to establish the relative contribution of sex chromosomes and hormones in sexual differentiation as

well as the interaction between the two. Arnold and Chen recently reviewed this model [109]. Here we highlight some of the model's basic concepts.

Figure 3 depicts the effect of the presence or absence of *Sry*—a 12kb region on the Y chromosome that is responsible for testis determination—using the FCG model. An XY mouse should develop testes; however, if *Sry* is deleted from the Y chromosome (symbolized by Y^-) then the mouse will develop ovaries [110]. If the *Sry* gene is inserted into any chromosome of an XX mouse (symbolized by XX(*Sry*+), then the mouse will develop testes. Finally, if *Sry* is deleted from the Y chromosome of an XY mouse and then inserted into one of its autosomes symbolized by XY^-(*Sry*+), then it will develop testes.

XY^-(*Sry*+) mice are fully fertile because the presence of *Sry* promotes testes development. XX(*Sry*+) mice lack some of the genes required for sperm production, which are found on the Y chromosome [111], and therefore do not appear to be fertile. However, they have small testes and are fully masculinized in terms of measures of male copulatory behavior, social exploration behavior, and sexually dimorphic neuroanatomical structures in the septum and lumbar spinal cord.

XY^-(*Sry*+) mice can be mated with XX females to produce the four types of offspring: XX, XY^-, XX(*Sry*+), and XY^-(*Sry*+) that can then be used to assess the impact of a mouse's chromosomal and gonadal sex on different phenotypes. For example, if there is a difference between mice that carry the *Sry* gene [i.e., XX(*Sry*+) and XY^-(*Sry*+)] versus those that do not (i.e., XX and XY^-), then the observed difference can be attributed to the gonadal type and/or presence of *Sry*. On the other hand, if there is a difference between mice that have the Y

chromosome [i.e., XY^{-} and XY^{-}(*Sry*+)] versus those that do not [i.e., XX and XX(*Sry*+)], then the observed difference can be attributed to complement of sex chromosomes (XX versus XY).

Some possible limits to the FCG model have been suggested. One possible limit is that the size, morphology, and function of the gonads are not exactly the same in XX and XY mice of the same gonadal type [e.g. XX(*Sry*+) vs. XY^{-} (*Sry*+)]. Consequently, the level of gonadal hormone secretions in FCG mice may differ during critical periods of development—a confound that has yet to be investigated. Nonetheless, numerous phenotypes that are responsive to the organizational effects of gonadal hormones (including sexually dimorphic brain structures) do not differ in XX and XY mice of same gonadal type [37,57,80,42], indicating that XX and XY mice of the same sex are likely experiencing similar levels of gonadal secretions. For example, measurements of circulating testosterone were not different in XX and XY males [112].

A second limitation relates to the biochemical and molecular environment. That is, one cannot rule out the effect of prenatal hormonal secretions, the influence of adult circulating hormones produced by the gonads/other tissues, or the acute fluctuations in hormonal levels.

To address these limits, it is best to rule out the effect of circulating gonadal hormones. An effective approach would be to first gonadectomize the mice followed by an administration of equivalent doses of gonadal steroid hormones. This is particularly important in the case of XY^{-} females since their level of ovarian steroid hormones differ from that in the XX wild type females [112]. Nevertheless, a major limitation still remains; it will not be obvious whether the sex difference attributed to the complement of sex chromosomes within cells is caused by (a) gene or genes encoded on the Y chromosome; (b) higher dosage of X genes particularly the ones that escape X inactivation in XX animals [113]; or (c) the parental imprints of the genes encoded

on the X chromosome in XX animals [61, 114]. If one determines that the sex difference in phenotype is due to the sex chromosome complement, then the next step would be to discover the nature of the gene or genes involved and identify whether those genes are encoded on the X or Y chromosome and how and where they mediate their role [115].

Notwithstanding these potential limitations, a variety of sex differences have been examined using the FCG model. We review four of these.

i. Dopaminergic neuron development

The notion that a genetic component could potentially contribute to brain sex differences was further strengthened in a study utilizing the four core genotype mouse model. Carruth et al. cultured mesencephalic neurons from E14 animals obtained from each group of the four core genotypes [116]. Cultures from XY^{-} and XY^{-}(*Sry*+) animals comprised significantly more dopaminergic, TH-ir neurons compared to the XX and XX(*Sry*+) animals indicating that genetic sex of the cells appears to be the major factor affecting the phenotype. In addition, in cultures representing animals that had *Sry* (and hence testes) a greater number of TH-ir cells was observed compared to those without *Sry* indicating that gonadal sex could also partially contribute to some of these differences. It is important to note the difficulty in separating the direct effects of *Sry* from its indirect effects (effects mediated through testis formation and the subsequent testosterone secretion) in the FCG mouse model.

ii. Lateral septum

One clear example of the role of sex-chromosome genes in brain phenotypes can be found in the lateral septum. The lateral septum is part of the limbic system and is involved in stress-

related behaviors. This nucleus is denser in male brains compared to female brains. However, it was found that the vasopressin fiber density was greater in the lateral septum of XY^{-}(*Sry*+) and XY^{-} mice compared to XX and XX(*Sry*+) mice [117]. In addition, an examination of vasopressin fiber densities in animals with the same sex chromosome complement indicated a role for the action of gonadal steroid hormones. No interaction was observed between gonadal sex and sex chromosomes [115].

iii. *Addiction*

On average, women use addictive drugs at lower levels than men, but women become addicted to drugs more rapidly than men [118]. Indeed, Quinn et al. using the FCG mouse model showed that sex differences in habit formation could be attributed to the differences in the complement of the sex chromosomes and not just to the gonadal secretions and/or the expression of the *Sry* gene. They found that XX mice developed habitual behavior more rapidly than the XY animals independent of their gonadal phenotype [119]. Their data demonstrated that sex chromosome complement could also play a role in the rate of habit formation in mice.

iv. *Aggression*

Males typically exhibit more aggressive behaviors compared to females [120-122]. Recent reports have shown that aggression latencies are strongly influenced by the simultaneous action of gonadal hormones and sex chromosomes. Using the four core genotypes model, it was found that a significant interaction exists between the two variables. In this model, the XX females appeared to be slower at displaying aggressive behavior on their first encounter with an intruder compared to animals in all other groups [117].

v. *Neural tube defects*

Anterior neural tube defects are more frequently observed in females than males [123]. These defects result in a wide range of developmental anomalies from spina bifida to exencephaly, anencephaly, to oral-facial midline defects [124]. Hundreds of genes are implicated in the regulation of neural tube closure (e.g., genes involved in cell proliferation, apoptosis, movement, and cell-cell adhesion) [125, 126]. Interestingly, mice with deletions in the tumor suppressor gene p53 display sex differences in anterior neural tube defects [126], with females experiencing more abnormalities than males, to the degree that no p53 null females survive on the first day of birth [127]. Using p53 null FCG mice, it was shown that XX mice of both gonadal type experience higher rates of mortality compared to XY mice, and that this difference was due to an X chromosome effect. Aberrant X-inactivation due to p53 deletions was suggested as a potential mechanism underlying the higher susceptibility of XX relative to XY cells to neural closure defects [127, 128].

B. The Sex Chromosome Trisomy (SCT) Model: An Experimental Model for Klinefelter Syndrome.

Besides the FCG mice, other mouse models such as those with sex chromosome anomalies (XXY, XO) could also provide an invaluable tool in advancing our understanding of direct genetic effects. To complement our FCG studies, we made use of an animal model of Klinefelter syndrome (KS). KS is the most frequent sex chromosome aneuploidy occurring in approximately 1:500 to 1:1000 live male births. It is the most common genetic cause of male infertility, arising from chromosomal nondisjunction during gametogenesis. The affected individuals have an extra X chromosome which could either be maternal or paternal in origin. XXY males manifest a wide

range of phenotypes including: tall stature, infertility, absence of germ cells, hypogonadism, gynecomastia, higher gonadotropin levels, serum testosterone deficiency and lower libido possibly associated with low serum testosterone levels [129, 130].

KS patients also exhibit impairments in social cognitive processing and executive functions. For example, many have speech development delay, learning disabilities, social impairments, emotional disregulation, poor executive functioning, and motor impairments. They are also at increased risk for lupus, autism, and attention deficit hyperactivity disorder (ADHD) [131]. In recent years, there has been growing interest in cognitive and behavioral impairments in KS following reports indicating that men with Klinefelter are at increased risk for developing schizophrenia or affective psychotic disorders [132, 133]. Men affected with KS seem to experience social interaction complications and display emotional difficulties [134]. Social deficits present themselves as introversion, anxiety, impulsive behavior, social withdrawal and timidity [135]. Boys with KS report having few to no friends, few to no interests or hobbies, and bad relationships with their family members during early adulthood [136]. In fact, in a recent report the extent to which KS men engage in certain social behaviors and the degree to which their social behavioral deficits contribute to autistic features have been quantitatively measured. Klinefelter men showed increased levels of distress while expressing negative feelings towards and initiating interaction with others compared to control men. They also seemed to participate less in several social behaviors and scored higher in a broad range of autistic traits [137]. Additionally, they often find it hard to comprehend their own emotions and exhibit a higher emotional arousal in response to emotional events. In addition, emotions seem to play a crucial role in strategic decision-making of the KS men [134].

In addition, some population-based reports suggest that men with KS are more inclined toward homosexuality than are other men, avoid social interaction, have more difficulty connecting with their male peers, are more likely to report distress about their assigned male gender identity and have low sex drive ([138, 139].

Since KS men suffer from multiple endocrine and neuropsychological complications, identifying the molecular mechanisms responsible for the KS phenotype is of particular interest. Both testicular failure (lower levels of androgens during puberty) and X gene overdosage may contribute to the clinical syndrome. Certainly research on experimental XXY animal models will inform the understanding of KS pathophysiology. In the mouse model developed by Arthur P. Arnold (University of California Los Angeles) and Paul S. Burgoyne (National Institute for Medical Research, UK)—hereafter referred to as the sex chromosome trisomy (SCT) model; transgenic XY*Sry* males are crossed with XXY^- females and eight genotypes are produced: XX, XXY^-, XY^-, XY^-Y^- females and XX*Sry*, XXY^-(*Sry*+), XY^-(*Sry*+), XY^-Y^-(*Sry*+) males. This animal model provides several advantages over the other animal models of KS: first is the ability to generate XXY mice in fewer generation numbers whereas other XXY models (e.g., the model developed by Ronald Swerdloff's group [24-26]) require a multiple-generation breeding scheme to produce these mice; secondly, using this model, one can tease apart the effects of gonadal status/hormones from sex chromosome effects. Lastly, multiple comparisons can be made 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 to examine if there are any interactions between these different factors and the gonadal sex.

Although the SCT mouse model of KS can be used for the more in-depth analyses of sex chromosome abnormalities, one needs to consider its caveats when interpreting results. For example, one very important question is whether the KS phenotype is due to the presence of the additional X chromosome, hormonal differences, gene expression alterations, parent of origin effects or some combination of all these factors. In our SCT model, hormonal levels are only measured during adulthood, but there is mounting evidence that differing gonadal hormone levels during the sensitive period can lead to differences in brain organization or that testosterone may have differing effects based on genotype. Therefore, one needs to be cautious when interpreting the origins of the observed KS-related phenotypes. In addition, parent-of-origin effects cannot be studied in the SCT mouse model because the breeding scheme that is used to generate the XXY mice always leads to each parent contributing one X chromosome to the XXY offspring ($X^{M}X^{P}$). This is not the case in human KS patients where only about half of KS men carry the maternal and paternal X chromosomes (as in SCT mice) and the other half have two X chromosomes of maternal origin. Furthermore, the ability to obtain sufficient animal numbers needed for complex experimental designs can sometimes be difficult in this mouse model.

Further investigations are necessary to fully characterize all the features of the SCT mice. For this reason, the evaluation of their metabolic profile is now underway in the laboratory of Dr. Arnold to ensure that this model recapitulates the pathology of KS.

However, it is important to note that despite all the limitations and pitfalls, the availability of this new model provides us with the opportunity to carry out previously impossible studies which will give us insight into the molecular and cellular mechanisms of this sex chromosome pathology.

Sexual Partner Preference

To examine how differences in the number of sex chromosomes can affect sexually dimorphic traits, we studied sexual partner preference in our mouse model of Klinefelter Syndrome (KS).

Sexual partner preference is perhaps one of the most sexually dimorphic traits between men and women, with most men being attracted to women, and vice-versa. However, approximately 2-6% of the human population has predominantly homosexual attractions [140]. Male sexuality is largely bimodal [141], with most men being either attracted to one sex or another (true male bisexuality appears to be rare), whereas female sexuality (often cited as being more fluid than that of a male's) has a more complicated distribution, with a much higher percentage having a bisexual orientation [142].

Neurological studies thus far have shown different hormonal and genetic factors affecting sexual orientation in males and females. Female homosexuality appears to be linked to androgen levels because women with congenital adrenal hyperplasia (CAH) have a significantly higher chance of being lesbian or bisexual. However, so far no studies have shown a direct association between an increased rate of homosexuality among men and androgen pathway disruptions (e.g. in hypovirilized XY individuals) [143] . While studies linking prenatal androgen's effects on sexual behavior in rats have been done, it has been difficult to draw solid conclusions about how well such findings correlate to human behavior because partner preference versus sexual behavior can be hard to distinguish in rodents (the act of lordosis does not necessarily indicate partner preference) [144]. A better model can be found in the domestic ram population, where 8-

10% of males prefer the same sex; such rams' coital behavior (mounting) is masculine [145], but their partner preference is "feminine", thus providing a much better distinction than rats.

So far brain studies have shown that homosexual men have a larger anterior commissure [146] and contain a larger arginine vasopressin neuronal population in their suprachiasmatic nucleus than control men and women [147], and a smaller INAH-3 than heterosexual males (similar in size to that of a female's) [148]. Analogous brain differences have been found in sheep as well. In addition, there has been additional evidence for a strong genetic factor influencing sexual orientation (at least in males). Perhaps the most well-known of these has been seen in family studies [149-152], with increased rates of homosexuality seen among siblings and maternal uncles of homosexual men [153], and there is a roughly 50% chance that both monozygotic twins are homosexual. Male homosexuality appears to be more linked to the mother's side of the family [141], and specifically, to the X-chromosome, in a region now identified as Xq28 [141]. Mothers with gay sons have also been found to have extreme skewing in regards to X-inactivation, compared to mothers with no gay sons [154]. Finally, birth order (with every older brother increasing one's chance of being homosexual by 33%) seems to point at the immune system as another possible determinant in sexual orientation, perhaps with the mother's body demonstrating stronger immunity against male-specific antigens with every male baby that she carries [155, 156].

AN EXPANDED VIEW ON SEX DIFFERENCES

Role of epigenetic modifications in brain sexual differentiation

The term 'epigenetic' refers to transmissible and stable changes in gene expression that are caused by mechanisms that do not involve a change in the nucleotide sequence. It refers to

functionally relevant chemical modifications to the genome that do not change the underlying DNA sequence. These changes consist of a vast array of chemical modifications that control the accessibility of the DNA to the transcription machinery. These modifications can modulate the activity of genes without altering their DNA sequence and are known to play a key role in channeling intrinsic and external signals into the genome.

Histone modifications and methylation of the cytosine residues in cytosine–guanine (CpG) dinucleotides reflect the best-known examples of these modifications [157]. DNA methylation catalyzed by a group of enzymes known as DNA methyltransferases (DNMTs) can govern the binding of regulatory proteins such as methyl CpG-binding domain proteins (MBDs). Once bound, they repress gene transcription upon recruitment of repressor complexes such as histone deacetylases (HDACs). These data indicate the presence of a crosstalk between DNA methylation marks and histone modifications in the control of gene transcription [158].

During brain development, sex differences have been reported in the activity of several components of the epigenetic machinery (e.g., epigenetic readers and writers such as MeCP2, and DNMTs, respectively) resulting in altered patterns of gene expression [31]. For instance, it has been shown that direct infusion of estradiol into the hippocampus of 12 week-old female mice increases mRNA and protein levels of certain DNMT enzymes (DNMT3a and DNMT3b), and leads to greater histone H3 acetylation and HDAC1 expression, but reduced HDAC2 levels [159]. Higher DNMT activity has also been observed in the POA of PN1 males relative to females, suggesting a DNMT-dependent role for gonadal hormones in producing sex differences in DNA methylation [31]. In addition to the hippocampus, MeCP2 expression appears to be elevated in the amygdala and the VMH of female rats only on PN1 and disappear by day 10 of

life in both brain regions, suggesting that the sex-specific changes in DNA methylation is more dynamic than previously thought [160]. However, it is noteworthy to mention that the transient sexually dimorphic expression of MeCP2 during a neonatal sensitive period indicates that MeCP2 is involved in brain sexual differentiation by refining the function of gonadal hormones. In fact, downregulating MeCP2 levels in the amygdala abolishes the sex dimorphism in prepubertal play behavior in both male and female rats during PN25–29 [161].

Apart from regulating the action of epigenetic readers, steroid hormones can dynamically regulate the expression of their own receptors through epigenetic modifications. For instance, DNA methylation of steroid receptors such as *ERα* greatly increases across development in several brain regions such as the cortex and the rat preoptic area (POA) and hypothalamus [162-164]. There is some evidence that *ERα* promoter methylation is higher in the male POA than in the female at PN10 resulting in reduced expression in the males, a finding that was corroborated by treatment of neonatal females with estradiol [reviewed in [30]]. Furthermore, higher AR expression in the cerebral cortex of adult and aged male mice has been associated with hormone-dependent lower AR promoter methylation [165]. These findings suggest that (1) perinatal exposure to gonadal steroids can establish sex-dependent alterations in hormone receptor gene DNA methylation resulting in long-lasting differences that could ultimately lead to sexual development; (2) these changes are tissue-specific and often transient and limited to particular developmental time windows.

Histone modifications are also implicated in brain sexual differentiation [166]. A recent study reported that levels of histones H3K9/14ac and H3K9me3 in the cortex and hippocampus of neonatal male mice are elevated as compared to females and that the increase in H3

acetylation levels can be induced in female mice with prenatal administration of testosterone to mimic the natural surge in testosterone occurring in males at embryonic days 17–19 [167]. Further support for the role of histone modification in brain sexual differentiation came from a study showing that postnatal day 1 injection of valproic acid (VPA), an HDAC inhibitor, prevents masculinization of the principal nucleus of the bed nucleus of the stria terminalis (BNSTp) in males and females treated with testosterone very early in life. Interestingly, both the changes in cell number and volume of the BNSTp remain until later in life [168].

Several recent studies have also investigated the role of microRNAs (miRs) in the context of brain sexual differentiation [169]. Tracy Bale and Christopher Morgan have identified a group of miRs with sexually dimorphic expression in postnatal day 2 whole brains of male and female mice [170]. Interestingly, the sex bias in the expression of almost half of the miRs investigated in their study appeared to be regulated by organizational effects of estradiol, and blocking testosterone conversion to estradiol with formestane in male mice during the neonatal period strongly disrupted this sex bias. Notably, in this study the sex-specific pattern of miR expression for a subset of genes was attributed to X gene dosage, suggesting the sex chromosome-dependent mechanisms also regulate the sex-biased expression of miRs [170].

Numerous miRs are also implicated in the cellular processes required for programming the sexually dimorphic brain, such as apoptosis [171]. For example, expression of miR-23a and its mRNA target, the X-linked inhibitor of apoptosis (XIAP), is sexually dimorphic causing differences in cell death activation pathways between the two sexes in a cerebral ischemia model [172]. Furthermore, it has been shown that estradiol induces expression of miR-101a and miR-199a in the developing preoptic area. These miRs regulate COX-2 and appear to be important for

sexual differentiation of dendritic spine density [173, 174]. These findings suggest a role for miRs in the establishment of sex differences in the brain.

Altogether, these findings suggest that epigenetics play a critical role in brain sexual differentiation and that changes in DNA methylation can be induced by gonadal hormones during different stages of brain development. Epigenetic mechanisms can work hand in hand with other signaling pathways to fine-tune gene expression, mediate hormonal effects during critical periods of brain sexual differentiation and lead to formation of sexually dimorphic brain structures that translate into permanent changes in brain function and behavior.

CONCLUSION

The overarching goal of this proposal is to identify the main determinants of brain sexual differentiation. In particular, the present research addresses the question of the contribution of sex chromosome genes to brain sex differences especially brain gene expression and investigates how differences in the number of sex chromosomes can affect sexually dimorphic behaviors such as partner preference. It also covers whether the effects of early hormone exposure on brain sexual differentiation is mediated through epigenetic mechanisms.

First, in using the Four Core Genotype mouse model to investigate the role of sex chromosome complement in the sexually dimorphic brain region of the striatum, several novel striatal genes are identified whose sex-differential expression is dependent on the sex chromosome complement rather than gonadal hormones (chapter 2).

Next, it is demonstrated (1) how highly sexually dimorphic traits such as partner preference can be feminized in a novel mouse model of KS (a chromosomal genetic condition characterized by the presence of an additional X chromosome) and (2) how this feminization

could potentially be related to the change in brain expression of several novel candidate genes that could be used in future studies to help explain some of the neurobehavioral phenotypes of KS, especially those that are feminized (chapter 3).

This proposal also explores the molecular basis of the organizational effects of testosterone and tests the hypothesis that the long term permanence may involve epigenetic mechanisms such as DNA methylation. To address this, comprehensive DNA methylation profiling is carried out for male (XY), female (XX), and female mice treated with testosterone (XX + T) and methylation maps are generated for the sexually dimorphic brain region of the striatum at two different time points: once during the sensitive period and once during adulthood. Resulting data demonstrate that testosterone contributes to both the establishment and maintenance of sex differences in methylation in the brain and testosterone can induce a broad shift in the DNA methylation status from a female-typical to a more male-typical pattern by day 60 of life. Data is presented here to show that the effect of testosterone on methylation is more pronounced during adulthood and that testosterone interacts with age to affect the methylation status of a large number of genes related to nervous system development and function. In addition, developmental stage (i.e., age) alone can also exhibit large effects on methylation. In this study, a subset of genes are identified that display differential methylation levels in response to testosterone but whose methylation levels are similar between males and females (genes exhibiting no sex-specific differences in methylation), suggesting that in certain contexts, testosterone effects on DNA methylation may serve to keep the two sexes as equivalent as possible, particularly in those genes that have comparable functions in males and females (chapter 4).

Ultimately, this work demonstrates the significance of genetics and epigenetics in the process of brain development as it relates to sex. It still confirms the hormonal theory of brain sexual differentiation, but offers a more comprehensive view that includes the interaction of genes, hormones, and epigenetics.

Table 1-1: Sex differences in behavioral traits in humans

Trait	Sex Bias	Evidence for the role of hormones	Evidence for the role of genetic factors	Other factors affecting sex differences in behavior
Cognition	Men do better at spatial tasks [175] and mathematical problem solving [176]. Women do better on verbal fluency, articulation, and verbal memory tests [177].	Prenatal hormone effects shown from studies of CAH, Turner's and androgen insensitivity syndromes [178]	No reliable evidence for the effect of sex chromosome genes proven from studies of Turner's and XX males [179]	Greater brain asymmetry in men for both verbal and non-verbal tasks [180, 181]
Play behavior-movement	There are sex differences in choice of toys, gender of the play partner, social play [182] and movement [183-185]	Testosterone influences juvenile play [186] Prenatal androgen levels affect play behavior and movement [187, 188]	Genetics sex seems to affect play behavior more than prenatal hormone exposure [186]	Parents and other socializing agents (i.e. peers, community, and child's own cognitive processes) [189] Developmental experience [190], visual information [191] affect movement organization
Language	Women perform better on episodic memory [192] and verbal fluency tasks, men are better at visuospatial processing [193-195] Greater dependence of females on declarative	Estrogen influences word and declarative memory abilities in women [198-206] Testosterone influences word memory in men [207] Prenatal testosterone levels relate to language processing in girls [208]	Single nucleotide polymorphisms in the gene, brain derived neurotrophic factor (BDNF) affecting BDNF secretion rates, partly accounting for greater dependence of females on declarative memory and the sex differences	Greater degrees of left hemispheric lateralization of brain for language in males and the bilateral language processing in females [210] Faster development of hippocampal brain regions in girls, activation of certain brain regions such as hippocampus and

	memory and males on procedural memory [196, 197]		observed in language-related tasks [209]	parahippocampal gyrus [211, 212]
Aggression	Foul language, imitation of aggressive models, violence and physical aggression more common in males [213]	Estradiol and progesterone influencing the serotonergic system [214, 215] Weak association between testosterone and aggression in both sexes [216, 217] High testosterone levels leading to increased verbal aggression and impulsivity in women [218, 219]	Association between serotonin transporter gene polymorphisms and greater impulsivity in males but not females [220] Polymorphisms in monoamine oxidase-A (MAOA) gene associated with antisocial personality disorder and aggression in males [221]	Low self-control, high impulsivity and negative emotionality [222] Sex-specific disparities in the neural circuitry of impulse control and emotion regulation, as well as serotonergic systems [223] Larger orbitofrontal cortexes in women [224]

Table 1-2: Sex differences in neurological disease.

Disease	Sex Bias	Evidence for the role of hormones	Evidence for the role of genetics	Other factors affecting sex differences in disease
Alzheimer's Disease (AD)	Women demonstrate higher AD prevalence at older ages ti[225, 226].	Gonadal hormones implicated in gender-related cognitive deficits of AD but the interaction is complex [227]	APOE allele type [228, 229] (i.e. Less and slower rate of amyloid plaque formation in men due to APOE ε2 [230])	Greater degeneration in areas of orbitofrontal cortex, middle and posterior cingulate cortex, hypothalamus, and mammilary bodies in men, and anterior thalamic in women [231].
Parkinson's disease (PD)	Overrepresented in males [232, 233] Age at onset is later in women [234]. Pathological symptoms of PD differ among males and females [235-237]	Most women manifest PD after menopause [238] Estrogen affecting BDNF secretion [239] Early life estrogen decline seems to be more important [240-242]	Linkage to X chromosome markers in 362 families, and to Xq28 in 443 discordant sibling pairs [243, 244] Val66met polymorphism in BDNF in women [245]	Environmental factors [246] Anatomical and structural differences in dopaminergic systems among males and females [189]
Autism	There is a high male to female ratio in the prevalence of autism [247]	Gonadal hormones affecting oxytocin (OT) and arginine vasopressin (AVP) receptors [248, 249]	Single nucleotide polymorphisms in the OT receptor in the Chinese Han [250] and American Caucasian population [251], SNPs in the vasopressin receptor (V1aR) gene [252, 253] X-chromosome	Alterations in oxytocin or arginine vasopressin activity, and differential processing of the oxytocin precursor [255-257]

			has effects on cognition and social aspects [130, 254]	
Addiction	Drug addiction more frequent in men [177] Higher relapse rates, faster progression of compulsive drug abuse and dependence have in women [258, 259])	Estradiol levels correlate with drug induced reinforcing behavior whereas progesterone levels are negatively associated with addiction [260-262]	Genes encoded on sex chromosomes can affect sex-related differences in addiction (the four core genotype mice) [119]	Neuroanatomical differences in motivation systems among males and females [189] Sex-related alterations in the cortico-limbic-striatal system that mediates reward processing [263]
Depression	Women are twice as likely as men to develop depression during reproductive years [264]	Low estrogen levels in female rats mediated by influences on neurotransmitter levels [265] Low testosterone levels associate with risk for depression in young and middle aged-men [266, 267]	Heritability rates estimated to be 70% [268] Polymorphisms in serotonin gene, estrogen receptor 1 (ESR1) polymorphism in the presence of Val/Val genotype of the Val158Met polymorphism in the Catechol-O-methyl transferase (COMT) gene, longer CA repeats of human estrogen receptor 2 (ESR2), short CAG repeats in androgen receptor gene [269]	Maladaptive coping, pessimism, dependency, low self- esteem, victimization, sexual abuse, comorbid anxiety disorder more common in depressed women [270] Early life events increase depression rates in adult women [271]

Anxiety disorders	The rate of anxiety disorders is higher in females [272]. The high co morbidity of these disorders with major depression helps account for the sex difference in depression [273] .	States of anxiety and panic have been reported to be affected by the menstrual cycle and pregnancy, implicating a role for estrogen and progesterone [272]. Pregnancy and lactation seem to alter brain neurochemical system that affect anxiety and fear [274].	The Val158 allele of COMT is associated with panic disorder in Caucasian women but not men [275]. In Asians, Met158 is associated with panic disorder in women but not men [275]. 5*HTT*LPR is a polymorphism associated with anxiety in humans. The orthologous polymorphism in rhesus macaques interacts with early adversity in a sexually dimorphic manner [276].	Animal studies indicate females undergo less neurobiological changes in response to stress compared to males [274]. It is speculated that this indicates increased adaptability in males and hence lower prevalence of affective illness [274].
Schizophrenia	more common in men than in women [277] Age at onset is later in women, another smaller peak of onset during peri- and post-menopause [277, 278] Pathological symptoms of schizophrenia differ among males and	This disease is not common before adolescence and puberty [280] Male schizophrenics have higher levels of Luteinizing Hormone (LH) and testosterone than healthy	Eight ultra-rare variants in eight distinct miRNA genes in 4% of analyzed males with schizophrenia [282] Relatives of females with schizophrenia demonstrate higher levels of	Anatomical and structural brain differences among males and females [279] Higher cortical levels in males as compared to females according to some studies [279] Higher sensitivity of the dopamine system in men as compared to women (Normal males produce

	females (males experience more negative symptoms, greater decrease in emotion expression and recognition,, greater paranoid delusions in women) [279] Lower chances of full recovery, and a poorer prognosis in men [277, 278] Anatomical brain differences between male and female patients	subjects, and female schizophrenics higher levels of LH and lower levels of estrogen [281]	the psychotic forms whereas relatives of schizophrenic men express lower rates of psychosis suggesting the presence of genetic heterogeneity [283] Higher rate of CAG repeat expansions among families of female patients and not male patients [284]	more striatal dopamine in response to an amphetamine challenge as compared to females) [285]

REFERENCES:

1. Ellegren, H. and J. Parsch, *The evolution of sex-biased genes and sex-biased gene expression.* Nature reviews. Genetics, 2007. **8**(9): p. 689-98.
2. Good, C.D., et al., *Cerebral Asymmetry and the Effects of Sex and Handedness on Brain Structure: A Voxel-Based Morphometric Analysis of 465 Normal Adult Human Brains.* NeuroImage, 2001. **14**(3): p. 685-700.
3. Matsumoto, A. and Y. Arai, *Male-female difference in synaptic organization of the ventromedial nucleus of the hypothalamus in the rat.* Neuroendocrinology, 1986. **42**(3): p. 232-6.
4. Witelson, S.F., Glezer, II, and D.L. Kigar, *Women have greater density of neurons in posterior temporal cortex.* J Neurosci, 1995. **15**(5 Pt 1): p. 3418-28.
5. Greenough, W.T., et al., *Sex differences in dentritic patterns in hamster preoptic area.* Brain Res, 1977. **126**(1): p. 63-72.
6. Ciofi, P., D. Leroy, and G. Tramu, *Sexual dimorphism in the organization of the rat hypothalamic infundibular area.* Neuroscience, 2006. **141**(4): p. 1731-1745.
7. Siddiqui, A. and B.H. Shah, *Neonatal androgen manipulation differentially affects the development of monoamine systems in rat cerebral cortex, amygdala and hypothalamus.* Brain Res Dev Brain Res, 1997. **98**(2): p. 247-52.
8. Nishizawa, S., et al., *Differences between males and females in rates of serotonin synthesis in human brain.* Proc Natl Acad Sci U S A, 1997. **94**(10): p. 5308-13.
9. Staley, J.K., et al., *Sex differences in [123I]beta-CIT SPECT measures of dopamine and serotonin transporter availability in healthy smokers and nonsmokers.* Synapse, 2001. **41**(4): p. 275-84.

10. Rhodes, M.E. and R.T. Rubin, *Functional sex differences ('sexual diergism') of central nervous system cholinergic systems, vasopressin, and hypothalamic-pituitary-adrenal axis activity in mammals: a selective review.* Brain Res Brain Res Rev, 1999. **30**(2): p. 135-52.

11. Hyde, J.S., *The gender similarities hypothesis.* Am Psychol, 2005. **60**(6): p. 581-92.

12. Lippa, R.A., *Sex differences in sex drive, sociosexuality, and height across 53 nations: testing evolutionary and social structural theories.* Arch Sex Behav, 2009. **38**(5): p. 631-51.

13. Schmitt, D.P., *Sociosexuality from Argentina to Zimbabwe: a 48-nation study of sex, culture, and strategies of human mating.* Behav Brain Sci, 2005. **28**(2): p. 247-75; discussion 275-311.

14. Sylvester, M. and S.C. Hayes, *Unpacking Masculinity as a Construct: Ontology, Pragmatism, and an Analysis of Language.* Psychology of Men & Masculinity, 2010. **11**(2): p. 91-97.

15. Brooks, G.R., *Despite Problems, "Masculinity" is a Vital Construct.* Psychology of Men & Masculinity, 2010. **11**(2): p. 107-108.

16. O'neil, J.M., *Is Criticism of Generic Masculinity, Essentialism, and Positive-Healthy-Masculinity a Problem for the Psychology of Men?* Psychology of Men & Masculinity, 2010. **11**(2): p. 98-106.

17. Harrison, P. and E. Tunbridge, *Catechol-O-Methyltransferase (COMT): A Gene Contributing to Sex Differences in Brain Function, and to Sexual Dimorphism in the Predisposition to Psychiatric Disorders.* Neuropsychopharmacology, 2008. **33**: p. 3037-3045.

18. Holden, C., *Sex and the suffering brain.* Science, 2005. **308**(5728): p. 1574.

19. Murray, H., et al., *Dose- and sex-dependent effects of the neurotoxin 6-hydroxydopamine on the nigrostriatal dopaminergic pathway of adult rats: differential actions of estrogen in males and females.* Neuroscience, 2003. **116**(213-222).

20. Baron-Cohen, S., R.C. Knickmeyer, and M.K. Belmonte, *Sex differences in the brain: implications for explaining autism.* Science, 2005. **310**(5749): p. 819-23.

21. Swerdlow, R.H., et al., *Gender ratio differences between Parkinson's disease patients and their affected relatives.* Parkinsonism & related disorders, 2001. **7**(2): p. 129-133.

22. Zhang, J.M., et al., *Impact of sex and hormones on new cells in the developing rat hippocampus: a novel source of sex dimorphism?* Eur J Neurosci, 2008. **27**(4): p. 791-800.

23. Arnold, A.P. and R.A. Gorski, *Gonadal steroid induction of structural sex differences in the central nervous system.* Annual review of neuroscience, 1984. **7**: p. 413-42.

24. Phoenix, C.H., et al., *Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig.* Endocrinology, 1959. **65**: p. 369-82.

25. McCarthy, M.M. and A.P. Arnold, *Reframing sexual differentiation of the brain.* Nature neuroscience, 2011. **14**(6): p. 677-83.

26. Arnold, A.P., *Experimental analysis of sexual differentiation of the zebra finch brain.* Brain research bulletin, 1997. **44**(4): p. 503-7.

27. Wade, J. and A.P. Arnold, *Functional testicular tissue does not masculinize development of the zebra finch song system.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(11): p. 5264-8.

28. Agate, R.J., et al., *Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(8): p. 4873-8.

29. Zhao, D., et al., *Somatic sex identity is cell autonomous in the chicken.* Nature, 2010. **464**(7286): p. 237-42.

30. Nugent, B.M. and M.M. McCarthy, *Epigenetic underpinnings of developmental sex differences in the brain.* Neuroendocrinology, 2011. **93**(3): p. 150-8.

31. McCarthy, M.M., et al., *The epigenetics of sex differences in the brain.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2009. **29**(41): p. 12815-23.

32. Lillie, F.R., *The Theory of the Free-Martin.* Science, 1916. **43**(1113): p. 611-3.

33. Jost, A., *Hormonal factors in the sex differentiation of the mammalian foetus.* Philosophical transactions of the Royal Society of London. Series B, Biological sciences, 1970. **259**(828): p. 119-30.

34. Jost, A., et al., *Studies on sex differentiation in mammals.* Recent progress in hormone research, 1973. **29**: p. 1-41.

35. Honda, S., et al., *Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene.* Biochemical and biophysical research communications, 1998. **252**(2): p. 445-9.

36. Ogawa, S., et al., *Abolition of male sexual behaviors in mice lacking estrogen receptors alpha and beta (alpha beta ERKO).* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(26): p. 14737-41.

37. Ogawa, S., et al., *Roles of estrogen receptor-alpha gene expression in reproduction-related behaviors in female mice.* Endocrinology, 1998. **139**(12): p. 5070-81.

38. Kudwa, A.E., et al., *A previously uncharacterized role for estrogen receptor beta: defeminization of male brain and behavior.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(12): p. 4608-12.

39. Juntti, S.A., et al., *The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors.* Neuron, 2010. **66**(2): p. 260-72.

40. Davis, E.C., P. Popper, and R.A. Gorski, *The role of apoptosis in sexual differentiation of the rat sexually dimorphic nucleus of the preoptic area.* Brain research, 1996. **734**(1-2): p. 10-8.

41. Waters, E.M. and R.B. Simerly, *Estrogen induces caspase-dependent cell death during hypothalamic development.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2009. **29**(31): p. 9714-8.

42. Krishnan, S., et al., *Central role of TRAF-interacting protein in a new model of brain sexual differentiation.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(39): p. 16692-7.

43. Wright, C.L., S.R. Burks, and M.M. McCarthy, *Identification of prostaglandin E2 receptors mediating perinatal masculinization of adult sex behavior and neuroanatomical correlates.* Developmental neurobiology, 2008. **68**(12): p. 1406-19.

44. Snyder, E.M., et al., *Role for A kinase-anchoring proteins (AKAPS) in glutamate receptor trafficking and long term synaptic depression.* The Journal of biological chemistry, 2005. **280**(17): p. 16962-8.

45. Wright, C.L. and M.M. McCarthy, *Prostaglandin E2-induced masculinization of brain and behavior requires protein kinase A, AMPA/kainate, and metabotropic glutamate receptor signaling.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2009. **29**(42): p. 13274-82.

46. McCarthy, M.M., *How it's made: organisational effects of hormones on the developing brain.* Journal of neuroendocrinology, 2010. **22**(7): p. 736-42.

47. Mong, J.A., E. Glaser, and M.M. McCarthy, *Gonadal steroids promote glial differentiation and alter neuronal morphology in the developing hypothalamus in a regionally specific manner.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 1999. **19**(4): p. 1464-72.

48. Todd, B.J., J.M. Schwarz, and M.M. McCarthy, *Prostaglandin-E2: a point of divergence in estradiol-mediated sexual differentiation.* Hormones and behavior, 2005. **48**(5): p. 512-21.

49. Schwarz, J.M., et al., *Estradiol induces hypothalamic dendritic spines by enhancing glutamate release: a mechanism for organizational sex differences.* Neuron, 2008. **58**(4): p. 584-98.

50. Breedlove, S.M. and A.P. Arnold, *Hormone accumulation in a sexually dimorphic motor nucleus of the rat spinal cord.* Science, 1980. **210**(4469): p. 564-6.

51. Breedlove, S.M., et al., *Masculinization of the female rat spinal cord following a single neonatal injection of testosterone propionate but not estradiol benzoate.* Brain research, 1982. **237**(1): p. 173-81.

52. Breedlove, S.M. and A.P. Arnold, *Sexually dimorphic motor nucleus in the rat lumbar spinal cord: response to adult hormone manipulation, absence in androgen-insensitive rats.* Brain research, 1981. **225**(2): p. 297-307.

53. Gotsiridze, T., et al., *Development of sex differences in the principal nucleus of the bed nucleus of the stria terminalis of mice: role of Bax-dependent cell death.* Developmental neurobiology, 2007. **67**(3): p. 355-62.

54. Fitch, R.H., et al., *Corpus callosum: effects of neonatal hormones on sexual dimorphism in the rat.* Brain research, 1990. **515**(1-2): p. 111-6.

55. Schulz, K.M., H.A. Molenda-Figueira, and C.L. Sisk, *Back to the future: The organizational-activational hypothesis adapted to puberty and adolescence.* Hormones and behavior, 2009. **55**(5): p. 597-604.

56. Arnold, A.P. and S.M. Breedlove, *Organizational and activational effects of sex steroids on brain and behavior: a reanalysis.* Hormones and behavior, 1985. **19**(4): p. 469-98.

57. Sisk, C.L. and J.L. Zehr, *Pubertal hormones organize the adolescent brain and behavior.* Frontiers in Neuroendocrinology, 2005. **26**(3-4): p. 163-74.

58. Menger, Y., et al., *Sex differences in brain epigenetics.* Epigenomics, 2010. **2**(6): p. 807-21.

59. Arnold, A.P., *Genetically triggered sexual differentiation of brain and behavior.* Horm Behav, 1996. **30**(4): p. 495-505.

60. Nottebohm, F. and A.P. Arnold, *Sexual dimorphism in vocal control areas of the songbird brain.* Science, 1976. **194**(4261): p. 211-3.

61. Pfaff, D.W., *Hormones, brain, and behavior*. Vol. 4. 2002, Amsterdam ; Boston: Academic Press.

62. Arnold, A.P., *The effects of castration on song development in zebra finches (Poephila guttata).* Journal of Experimental Zoology, 1975. **191**: p. 261-278.

63. Wade, J. and A.P. Arnold, *Functional testicular tissue does not masculinize development of the zebra finch song system.* Proc Natl Acad Sci U S A, 1996. **93**(11): p. 5264-8.

64. Wade, J., *Zebra finch sexual differentiation: the aromatization hypothesis revisited.* Microsc Res Tech, 2001. **54**(6): p. 354-63.

65. Gurney, M.E. and M. Konishi, *Hormone-Induced Sexual Differentiation of Brain and Behavior in Zebra Finches.* Science, 1980. **208**(4450): p. 1380-1383.

66. Jacobs, E.C., W. Grisham, and A.P. Arnold, *Lack of a synergistic effect between estradiol and dihydrotestosterone in the masculinization of the zebra finch song system.* J Neurobiol, 1995. **27**(4): p. 513-9.

67. Simpson, H.B. and D.S. Vicario, *Early estrogen treatment of female zebra finches masculinizes the brain pathway for learned vocalizations.* J Neurobiol, 1991. **22**(7): p. 777-93.

68. Arnold, A.P. and P.S. Burgoyne, *Are XX and XY brain cells intrinsically different?* Trends Endocrinol Metab, 2004. **15**(1): p. 6-11.

69. Dittrich, F., et al., *Estrogen-inducible, sex-specific expression of brain-derived neurotrophic factor mRNA in a forebrain song control nucleus of the juvenile zebra finch.* Proc Natl Acad Sci U S A, 1999. **96**(14): p. 8241-6.

70. Holloway, C.C. and D.F. Clayton, *Estrogen synthesis in the male brain triggers development of the avian song control pathway in vitro.* Nat Neurosci, 2001. **4**(2): p. 170-5.

71. Arnold, A.P., *Sexual differentiation of the zebra finch song system: positive evidence, negative evidence, null hypotheses, and a paradigm shift.* J Neurobiol, 1997. **33**(5): p. 572-84.

72. Arnold, A.P., et al., *Sexual differentiation of the brain in songbirds.* Dev Neurosci, 1996. **18**(1-2): p. 124-36.

73. Agate, R.J., et al., *Neural, not gonadal, origin of brain sex differences in a gynandromorphic finch.* Proc Natl Acad Sci U S A, 2003. **100**(8): p. 4873-8.

74. Patek, C.E., et al., *Sex chimaerism, fertility and sex determination in the mouse.* Development, 1991. **113**(1): p. 311-325.

75. Burgoyne, P.S., M. Buehr, and A. McLaren, *XY follicle cells in ovaries of XXâ€"XY female mouse chimaeras.* Development, 1988. **104**(4): p. 683-688.

76. O, W.S., et al., *Primary genetic control of somatic sexual differentiation in a mammal.* Nature, 1988. **331**(6158): p. 716-7.

77. Renfree, M.B. and R.V. Short, *Sex determination in marsupials: evidence for a marsupial-eutherian dichotomy.* Philos Trans R Soc Lond B Biol Sci, 1988. **322**(1208): p. 41-53.

78. Renfree, M.B., et al., *Sexual differentiation of the urogenital system of the fetal and neonatal tammar wallaby, Macropus eugenii.* Anat Embryol (Berl), 1996. **194**(2): p. 111-34.

79. Wilson, J.D., F.W. George, and M.B. Renfree, *The endocrine role in mammalian sexual differentiation.* Recent Prog Horm Res, 1995. **50**: p. 349-64.

80. Vilain, E., *Genetics of intersexuality.* Journal of Gay & Lesbian Psychotherapy, 2006. **10**(2): p. 9-26.

81. Lucas, J.C., et al., *The influence of the anti-androgen flutamide on early sexual differentiation of the marsupial male.* J Reprod Fertil, 1997. **109**(2): p. 205-12.

82. Shaw, G., et al., *Experimental manipulation of sexual differentiation in wallaby pouch young treated with exogenous steroids.* Development, 1988. **104**(4): p. 689-701.

83. Sharman, G.B., *The chromosomal basis of sex differentiation in marsupials.* Aust. J. Zool, 1990. **37**: p. 451-466.

84. Cooper, D.W., *The evolution of sex determination, sex chromosome dimorphism, and X-inactivation in therian mammals: A comparison of metatherians (marsupials) and eutherians ("placentals")*, in *Sex chromosomes and sex-determining genes*, K. Reed and J.A.M. Graves, Editors. 1993, Harwood Academic Publishers: Chur, Switzerland ; Langhorne, PA. p. 183-200.

85. Gibb, W.R., *Neuropathology of Parkinson's disease and related syndromes.* Neurol Clin, 1992. **10**(2): p. 361-76.

86. Reisert, I. and C. Pilgrim, *Sexual differentiation of monoaminergic neurons - genetic or epigenetic?* Trends in Neurosciences, 1991. **14**(10): p. 468-473.

87. Sibug, R., et al., *Genotype-dependent sex differentiation of dopaminergic neurons in primary cultures of embryonic mouse brain.* Brain Res Dev Brain Res, 1996. **93**(1-2): p. 136-42.

88. Mayer, A., et al., *The Y-chromosomal genes SRY and ZFY are transcribed in adult human brain.* Neurogenetics, 1998. **1**(4): p. 281-8.

89. Czech, D.P., et al., *The human testis-determining factor SRY localizes in midbrain dopamine neurons and regulates multiple components of catecholamine synthesis and metabolism.* Journal of neurochemistry, 2012.

90. Kim, K.S., et al., *Orphan nuclear receptor Nurr1 directly transactivates the promoter activity of the tyrosine hydroxylase gene in a cell-specific manner.* Journal of neurochemistry, 2003. **85**(3): p. 622-34.

91. Jin, H., et al., *Tyrosine hydroxylase gene regulation in human neuronal progenitor cells does not depend on Nurr1 as in the murine and rat systems.* Journal of cellular physiology, 2006. **207**(1): p. 49-57.

92. Mayer, A., et al., *Developmental profile of Sry transcripts in mouse brain.* Neurogenetics, 2000. **3**(1): p. 25-30.

93. Milsted, A., et al., *Regulation of tyrosine hydroxylase gene transcription by Sry.* Neuroscience letters, 2004. **369**(3): p. 203-7.

94. Dewing, P., et al., *Direct regulation of adult brain function by the male-specific factor SRY.* Curr Biol, 2006. **16**(4): p. 415-20.

95. Dewing, P., et al., *Direct regulation of adult brain function by the male-specific factor SRY.* Current biology : CB, 2006. **16**(4): p. 415-20.

96. Ely, D., et al., *Sry delivery to the adrenal medulla increases blood pressure and adrenal medullary tyrosine hydroxylase of normotensive WKY rats.* BMC cardiovascular disorders, 2007. **7**: p. 6.

97. Zhang, K., et al., *Human tyrosine hydroxylase natural genetic variation: delineation of functional transcriptional control motifs disrupted in the proximal promoter.* Circulation. Cardiovascular genetics, 2010. **3**(2): p. 187-98.

98. Tao, Q., et al., *Gender segregation in gene expression and vulnerability to oxidative stress induced injury in ventral mesencephalic cultures of dopamine neurons.* Journal of neuroscience research, 2012. **90**(1): p. 167-78.

99. Wu, J.B., et al., *Regulation of monoamine oxidase A by the SRY gene on the Y chromosome.* FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 2009. **23**(11): p. 4029-38.

100. Fernandez-Ruiz, J.J., et al., *Nigrostriatal and mesolimbic dopaminergic activities were modified throughout the ovarian cycle of female rats.* Journal of neural transmission. General section, 1991. **85**(3): p. 223-9.

101. Xiao, L. and J.B. Becker, *Quantitative microdialysis determination of extracellular striatal dopamine concentration in male and female rats: effects of estrous cycle and gonadectomy.* Neuroscience letters, 1994. **180**(2): p. 155-8.

102. Crowley, W.R., T.L. O'Donohue, and D.M. Jacobowitz, *Changes in catecholamine content in discrete brain nuclei during the estrous cycle of the rat.* Brain research, 1978. **147**(2): p. 315-26.

103. Jori, A. and G. Cecchetti, *Homovanillic acid levels in rat striatum during the oestrous cycle.* The Journal of endocrinology, 1973. **58**(2): p. 341-2.

104. Davis, C.F., B.F. Davis, and A.E. Halaris, *Variations in the uptake of 3H-dopamine during the estrous cycle.* Life sciences, 1977. **20**(8): p. 1319-32.

105. Morissette, M. and T. Di Paolo, *Sex and estrous cycle variations of rat striatal dopamine uptake sites.* Neuroendocrinology, 1993. **58**(1): p. 16-22.

106. Di Paolo, T., P. Falardeau, and M. Morissette, *Striatal D-2 dopamine agonist binding sites fluctuate during the rat estrous cycle.* Life sciences, 1988. **43**(8): p. 665-72.

107. Becker, J.B., *Gender differences in dopaminergic function in striatum and nucleus accumbens.* Pharmacology, biochemistry, and behavior, 1999. **64**(4): p. 803-12.

108. Pohjalainen, T., et al., *Sex differences in the striatal dopamine D2 receptor binding characteristics in vivo.* The American journal of psychiatry, 1998. **155**(6): p. 768-73.

109. Arnold, A.P. and X. Chen, *What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues?* Frontiers in Neuroendocrinology, 2009. **30**(1): p. 1-9.

110. Gubbay, J., et al., *Normal structure and expression of Zfy genes in XY female mice mutant in Tdy.* Development, 1990. **109**(3): p. 647-53.

111. McCarty, B.M., et al., *Medical and psychosexual outcome in women affected by complete gonadal dysgenesis.* J Pediatr Endocrinol Metab, 2006. **19**(7): p. 873-7.

112. Arnold, A.P. and X. Chen, *What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues?* Front Neuroendocrinol, 2009. **30**(1): p. 1-9.

113. Carrel, L., et al., *A first-generation X-inactivation profile of the human X chromosome.* Proc Natl Acad Sci U S A, 1999. **96**(25): p. 14440-4.

114. Xu, J., P.S. Burgoyne, and A.P. Arnold, *Sex differences in sex chromosome gene expression in mouse brain.* Hum Mol Genet, 2002. **11**(12): p. 1409-19.

115. De Vries, G.J., et al., *A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits.* J Neurosci, 2002. **22**(20): p. 9005-14.

116. Carruth, L.L., I. Reisert, and A.P. Arnold, *Sex chromosome genes directly affect brain sexual differentiation.* Nat Neurosci, 2002. **5**(10): p. 933-934.

117. Gatewood, J.D., et al., *Sex chromosome complement and gonadal sex influence aggressive and parental behaviors in mice.* J Neurosci, 2006. **26**(8): p. 2335-42.

118. Hart, C.L. and G.D. Smith, *Alcohol consumption and mortality and hospital admissions in men from the Midspan collaborative cohort study.* Addiction, 2008. **103**(12): p. 1979-86.

119. Quinn, J.J., et al., *Sex chromosome complement regulates habit formation.* Nat Neurosci, 2007. **10**(11): p. 1398-400.

120. Murphy, C.M., C.T. Taft, and C.I. Eckhardt, *Anger problem profiles among partner violent men: Differences in clinical presentation and treatment outcome.* Journal of Counseling Psychology, 2007. **54**(2): p. 189-200.

121. Cohn, A.M., A. Zeicher, and A.L. Seibert, *Labile affect as a risk factor for aggressive behavior in men.* Psyc Men Masc 2008. **9**: p. 29-39.

122. Tager, D., G.E. Good, and S. Brammer, *"Walking Over 'Em": An Exploration of Relations Between Emotion Dysregulation, Masculine Norms, and Intimate Partner Abuse in a Clinical Sample of Men.* Psychology of Men & Masculinity, 2010. **11**(3): p. 233-239.

123. Seller, M.J., *Sex, neural tube defects, and multisite closure of the human neural tube.* American journal of medical genetics, 1995. **58**(4): p. 332-6.

124. Copp, A.J., N.D. Greene, and J.N. Murdoch, *The genetic basis of mammalian neurulation.* Nature reviews. Genetics, 2003. **4**(10): p. 784-93.

125. Harris, M.J. and D.M. Juriloff, *Mouse mutants with neural tube closure defects and their role in understanding human neural tube defects.* Birth defects research. Part A, Clinical and molecular teratology, 2007. **79**(3): p. 187-210.

126. Armstrong, J.F., et al., *High-frequency developmental abnormalities in p53-deficient mice.* Current biology : CB, 1995. **5**(8): p. 931-6.

127. Chen, X., et al., *Sex difference in neural tube defects in p53-null mice is caused by differences in the complement of X not Y genes.* Developmental neurobiology, 2008. **68**(2): p. 265-73.

128. Cranston, A., et al., *Female embryonic lethality in mice nullizygous for both Msh2 and p53.* Nature genetics, 1997. **17**(1): p. 114-8.

129. Lanfranco, F., et al., *Klinefelter's syndrome.* Lancet, 2004. **364**(9430): p. 273-83.

130. Marco, E.J. and D.H. Skuse, *Autism-lessons from the X chromosome.* Soc Cogn Affect Neurosci, 2006. **1**(3): p. 183-193.

131. Kielinen, M., et al., *Associated medical disorders and disabilities in children with autistic disorder: a population-based study.* Autism : the international journal of research and practice, 2004. **8**(1): p. 49-60.

132. Delisi, L.E., et al., *Klinefelter's Syndrome (XXY) as a genetic model for psychotic disorders (vol 135B, pg 15, 2005).* American Journal of Medical Genetics Part B-Neuropsychiatric Genetics, 2005. **136B**(1): p. 107-107.

133. Bojesen, A., et al., *Morbidity in Klinefelter syndrome: A Danish register study based on hospital discharge diagnoses.* Journal of Clinical Endocrinology and Metabolism, 2006. **91**(4): p. 1254-1260.

134. Temple, C.A. and P.M. Sanfilippo, *Executive skills in Klinefelter's syndrome.* Neuropsychologia, 2003. **41**(11): p. 1547-1559.

135. Geschwind, D.H., et al., *Neurobehavioral phenotype of Klinefelter syndrome.* Mental Retardation and Developmental Disabilities Research Reviews, 2000. **6**(2): p. 107-116.

136. Nielsen, J., S.G. Johnsen, and K. Sorensen, *Follow-up 10 Years Later of 34 Klinefelter Males with Karyotype 47,Xxy and 16 Hypogonadal Males with Karyotype 46,Xy.* Psychological Medicine, 1980. **10**(2): p. 345-352.

137. van Rijn, S., et al., *Social Behavior and Autism Traits in a Sex Chromosomal Disorder: Klinefelter (47XXY) Syndrome.* J Autism Dev Disord, 2008.

138. Bancroft, J., D. Axworthy, and S. Ratcliffe, *The personality and psycho-sexual development of boys with 47 XXY chromosome constitution.* Journal of child psychology and psychiatry, and allied disciplines, 1982. **23**(2): p. 169-80.

139. Schiavi, R.C., et al., *Sex chromosome anomalies, hormones, and sexuality.* Archives of general psychiatry, 1988. **45**(1): p. 19-24.

140. Diamond, M., *Homosexuality and bisexuality in different populations.* Archives of sexual behavior, 1993. **22**(4): p. 291-310.

141. Hamer, D., et al., *A linkage between DNA markers on the X chromosome and male sexual orientation.* Science, 1993. **261**(5119): p. 321-327.

142. Hu, S., et al., *Linkage between sexual orientation and chromosome Xq28 in males but not females.* Nature Genetics, 1995. **11**: p. 248-256.

143. Hines, M., C. Brook, and G.S. Conway, *Androgen and psychosexual development: core gender identity, sexual orientation and recalled childhood gender role behavior in women and men with congenital adrenal hyperplasia (CAH).* J Sex Res, 2004. **41**(1): p. 75-81.

144. Ngun, T.C., et al., *The genetics of sex differences in brain and behavior.* Frontiers in Neuroendocrinology, 2011. **32**(2): p. 227-46.

145. Roselli, C.E. and F. Stormshak, *The neurobiology of sexual partner preferences in rams.* Horm Behav, 2009. **55**(5): p. 611-20.

146. Allen, L.S. and R.A. Gorski, *Sexual orientation and the size of the anterior commissure in the human brain.* Proceedings of the National Academy of Sciences of the US, 1992. **89**: p. 7199-7202.

147. Swaab, D.F., et al., *Sexual differentiation of the human hypothalamus: Differences according to sex, sexual orientation, and transsexuality*, in *Ellis, Lee (Ed); Ebertz, Linda (Ed). (1997). Sexual orientation: Toward biological understanding. (pp. 129 150). Westport, CT, US: Praeger Publishers/Greenwood Publishing Group, Inc. xxii, 276 pp.SEE BOOK*. 1997.

148. LeVay, S., *A difference in hypothalamic structure between heterosexual and homosexual men.* Science, 1991. **253**: p. 1034-1037.

149. Pillard, R.C. and J.D. Weinrich, *Evidence of familial nature of male homosexuality.* Archives of General Psychiatry, 1986. **43**: p. 808-812.

150. Bailey, J.M. and R.C. Pillard, *A genetic study of male sexual orientation.* Archives of General Psychiatry, 1991. **48**(12): p. 1089-1096.

151. Pattatucci, A.M.L. and D.H. Hamer, *Development and familiality of sexual orientation in females.* Behavior Genetics, 1995. **25**(5): p. 407-420.

152. Bailey, J.M., et al., *A family history study of male sexual orientation using three independent samples.* Behavior Genetics, 1999. **29**(2): p. 79-86.

153. Bailey, J.M. and R.C. Pillard, *Genetics of human sexual orientation.* Annual Review of Sex Research, 1995: p. 126-150.

154. Bocklandt, S., et al., *Extreme skewing of X chromosome inactivation in mothers of homosexual men.* Hum Genet, 2006. **118**(6): p. 691-4.

155. Blanchard, R., *Birth order and sibling sex ration in homosexual versus heterosexual males and females.* Annual Review of Sex Research, 1997. **8**: p. 27-67.

156. Jones, M.B. and R. Blanchard, *Birth order and male homosexuality: extension of Slater's index.* Hum Biol, 1998. **70**(4): p. 775-87.

157. Reik, W., *Stability and flexibility of epigenetic gene regulation in mammalian development.* Nature, 2007. **447**(7143): p. 425-32.

158. Goldberg, A.D., C.D. Allis, and E. Bernstein, *Epigenetics: a landscape takes shape.* Cell, 2007. **128**(4): p. 635-8.

159. Zhao, Z., L. Fan, and K.M. Frick, *Epigenetic alterations regulate estradiol-induced enhancement of memory consolidation.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(12): p. 5605-10.

160. Kurian, J.R., R.M. Forbes-Lorman, and A.P. Auger, *Sex difference in mecp2 expression during a critical period of rat brain development.* Epigenetics : official journal of the DNA Methylation Society, 2007. **2**(3): p. 173-8.

161. Kurian, J.R., et al., *Mecp2 organizes juvenile social behavior in a sex-specific manner.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2008. **28**(28): p. 7137-42.

162. Westberry, J.M., A.L. Trout, and M.E. Wilson, *Epigenetic regulation of estrogen receptor alpha gene expression in the mouse cortex during early postnatal development.* Endocrinology, 2010. **151**(2): p. 731-40.

163. Schwarz, J.M., B.M. Nugent, and M.M. McCarthy, *Developmental and hormone-induced epigenetic changes to estrogen and progesterone receptor genes in brain are dynamic across the life span.* Endocrinology, 2010. **151**(10): p. 4871-81.

164. Kurian, J.R., K.M. Olesen, and A.P. Auger, *Sex differences in epigenetic regulation of the estrogen receptor-alpha promoter within the developing preoptic area.* Endocrinology, 2010. **151**(5): p. 2297-305.

165. Kumar, R.C. and M.K. Thakur, *Androgen receptor mRNA is inversely regulated by testosterone and estradiol in adult mouse brain.* Neurobiology of aging, 2004. **25**(7): p. 925-33.

166. Matsuda, K.I., et al., *Histone Deacetylation during Brain Development Is Essential for Permanent Masculinization of Sexual Behavior.* Endocrinology, 2011. **152**(7): p. 2760-7.

167. Tsai, H.W., P.A. Grant, and E.F. Rissman, *Sex differences in histone modifications in the neonatal mouse brain.* Epigenetics : official journal of the DNA Methylation Society, 2009. **4**(1): p. 47-53.

168. Murray, E.K., et al., *Epigenetic control of sexual differentiation of the bed nucleus of the stria terminalis.* Endocrinology, 2009. **150**(9): p. 4241-7.

169. Morgan, C.P. and T.L. Bale, *Sex differences in microRNA regulation of gene expression: no smoke, just miRs.* Biology of sex differences, 2012. **3**(1): p. 22.

170. Morgan, C.P. and T.L. Bale, *Early prenatal stress epigenetically programs dysmasculinization in second-generation offspring via the paternal lineage.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2011. **31**(33): p. 11748-55.

171. Jovanovic, M. and M.O. Hengartner, *miRNAs and apoptosis: RNAs to die for.* Oncogene, 2006. **25**(46): p. 6176-87.

172. Siegel, C., et al., *miR-23a regulation of X-linked inhibitor of apoptosis (XIAP) contributes to sex differences in the response to cerebral ischemia.* Proceedings of the National Academy of Sciences of the United States of America, 2011. **108**(28): p. 11662-7.

173. Chakrabarty, A., et al., *MicroRNA regulation of cyclooxygenase-2 during embryo implantation.* Proceedings of the National Academy of Sciences of the United States of America, 2007. **104**(38): p. 15144-9.

174. Amateau, S.K. and M.M. McCarthy, *A novel mechanism of dendritic spine plasticity involving estradiol induction of prostaglandin-E2.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2002. **22**(19): p. 8586-96.

175. Voyer, D., S. Voyer, and M.P. Bryden, *Magnitude of sex differences in spatial abilities: a meta-analysis and consideration of critical variables.* Psychol Bull, 1995. **117**(2): p. 250-70.

176. Benbow, C.P., et al., *Sex differences in mathematical reasoning ability at age 13: their status 20 years later.* Psychol Sci, 2000. **11**(6): p. 474-80.

177. Becker, J.B., *Sex differences in the brain : from genes to behavior*. 2008, Oxford ; New York: Oxford University Press. xxiv, 480 p.

178. Halpern, D.F., *Sex Differences in Cognitive Abilities*. 2000, Lawrence Erlbaum Associates. p. 159-163.

179. Halpern, D.F., *Sex Differences in Cognitive Abilities*. 2000, Lawrence Erlbaum Associates. p. 147-148.

180. Harshman, R.A., E. Hampson, and S.A. Berenbaum, *Individual differences in cognitive abilities and brain organization, Part I: Sex and handedness differences in ability.* Can J Psychol, 1983. **37**(1): p. 144-92.

181. Kimura, D. and R.A. Harshman, *Sex differences in brain organization for verbal and non-verbal functions.* Prog Brain Res, 1984. **61**: p. 423-41.

182. Hines, M., *Brain gender*. 2004, Oxford ; New York: Oxford University Press. xiii, 307 p.

183. Almli, C.R., R.H. Ball, and M.E. Wheeler, *Human fetal and neonatal movement patterns: Gender differences and fetal-to-neonatal continuity.* Dev Psychobiol, 2001. **38**(4): p. 252-73.

184. Davies, P.L. and J.D. Rose, *Motor skills of typically developing adolescents: awkwardness or improvement?* Phys Occup Ther Pediatr, 2000. **20**(1): p. 19-42.

185. Piek, J.P., et al., *Limb and gender differences in the development of coordination in early infancy.* Hum Mov Sci, 2002. **21**(5-6): p. 621-39.

186. Auyeung, B., et al., *Fetal testosterone predicts sexually differentiated childhood behavior in girls and in boys.* Psychol Sci, 2009. **20**(2): p. 144-8.

187. Nordenstrom, A., et al., *Sex-typed toy play behavior correlates with the degree of prenatal androgen exposure assessed by CYP21 genotype in girls with congenital adrenal hyperplasia.* J Clin Endocrinol Metab, 2002. **87**(11): p. 5119-24.

188. Berenbaum, S.A., S.C. Duck, and K. Bryk, *Behavioral effects of prenatal versus postnatal androgen excess in children with 21-hydroxylase-deficient congenital adrenal hyperplasia.* J Clin Endocrinol Metab, 2000. **85**(2): p. 727-33.

189. Becker, J.B., *Sex differences in the brain : from genes to behavior*. 2008, Oxford University Press: Oxford ; New York.

190. Hall, J.A. and D. Kimura, *Sexual orientation and performance on sexually dimorphic motor tasks.* Arch Sex Behav, 1995. **24**(4): p. 395-407.

191. Tottenham, L.S. and D.M. Saucier, *Throwing accuracy during prism adaptation: male advantage for throwing accuracy is independent of prism adaptation rate.* Percept Mot Skills, 2004. **98**(3 Pt 2): p. 1449-55.

192. Herlitz, A., E. Airaksinen, and E. Nordstrom, *Sex differences in episodic memory: The impact of verbal and visuospatial ability.* Neuropsychology, 1999. **13**(4): p. 590-597.

193. Kimura, D., *Sex, sexual orientation and sex hormones influence human cognitive function.* Curr Opin Neurobiol, 1996. **6**(2): p. 259-63.

194. Lewin, C., G. Wolgers, and A. Herlitz, *Sex differences favoring women in verbal but not in visuospatial episodic memory.* Neuropsychology, 2001. **15**(2): p. 165-73.

195. Sherwin, B.B., *Estrogen and cognitive functioning in women.* Endocr Rev, 2003. **24**(2): p. 133-51.

196. Ullman, M.T., *Contributions of memory circuits to language: the declarative/procedural model.* Cognition, 2004. **92**(1-2): p. 231-70.

197. Hartshorne, J.K. and M.T. Ullman, *Why girls say 'holded' more than boys.* Dev Sci, 2006. **9**(1): p. 21-32.

198. Sherwin, B.B., *Estrogen and cognitive functioning in women.* Proc Soc Exp Biol Med, 1998. **217**(1): p. 17-22.

199. Phillips, S.M. and B.B. Sherwin, *Effects of estrogen on memory function in surgically menopausal women.* Psychoneuroendocrinology, 1992. **17**(5): p. 485-95.

200. Maki, P.M. and S.M. Resnick, *Longitudinal effects of estrogen replacement therapy on PET cerebral blood flow and cognition.* Neurobiol Aging, 2000. **21**(2): p. 373-83.

201. Simpkins, J.W., et al., *Role of estrogen replacement therapy in memory enhancement and the prevention of neuronal loss associated with Alzheimer's disease.* Am J Med, 1997. **103**(3A): p. 19S-25S.

202. Packard, M.G., *Posttraining estrogen and memory modulation.* Horm Behav, 1998. **34**(2): p. 126-39.

203. Shughrue, P.J., P.J. Scrimo, and I. Merchenthaler, *Estrogen binding and estrogen receptor characterization (ERalpha and ERbeta) in the cholinergic neurons of the rat basal forebrain.* Neuroscience, 2000. **96**(1): p. 41-9.

204. Murphy, D.D., N.B. Cole, and M. Segal, *Brain-derived neurotrophic factor mediates estradiol-induced dendritic spine formation in hippocampal neurons.* Proc Natl Acad Sci U S A, 1998. **95**(19): p. 11412-7.

205. Woolley, C.S., *Effects of estrogen in the CNS.* Curr Opin Neurobiol, 1999. **9**(3): p. 349-54.

206. Scharfman, H.E. and N.J. Maclusky, *Similarities between actions of estrogen and BDNF in the hippocampus: coincidence or clue?* Trends Neurosci, 2005. **28**(2): p. 79-85.

207. Estabrooke, I.V., et al., *The effect of sex hormones on language processing.* Brain and Language, 2002. **83**(1): p. 143-146.

208. Grimshaw, G.M., M.P. Bryden, and J.A.K. Finegan, *Relations between Prenatal Testosterone and Cerebral Lateralization in Children.* Neuropsychology, 1995. **9**(1): p. 68-79.

209. Egan, M.F., et al., *The BDNF val66met polymorphism affects activity-dependent secretion of BDNF and human memory and hippocampal function.* Cell, 2003. **112**(2): p. 257-69.

210. Kansaku, K., A. Yamaura, and S. Kitazawa, *Sex differences in lateralization revealed in the posterior language areas.* Cereb Cortex, 2000. **10**(9): p. 866-72.

211. Pfluger, T., et al., *Normative volumetric data of the developing hippocampus in children based on magnetic resonance imaging.* Epilepsia, 1999. **40**(4): p. 414-23.

212. Becker, J.B., *Sex differences in the brain : from genes to behavior*. 2008, Oxford University Press: Oxford ; New York. p. 427.

213. Bettencourt, B.A. and N. Miller, *Gender differences in aggression as a function of provocation: A meta-analysis.* Psychological Bulletin, 1996. **119**(3): p. 422-447.

214. Wihlback, A.C., et al., *Influence of menstrual cycle on platelet serotonin uptake site and serotonin(2A) receptor binding.* Psychoneuroendocrinology, 2004. **29**(6): p. 757-766.

215. Rubinow, D.R., P.J. Schmidt, and C.A. Roca, *Estrogen-serotonin interactions: Implications for affective regulation.* Biological Psychiatry, 1998. **44**(9): p. 839-850.

216. Archer, J., N. Graham-Kevan, and M. Davies, *Testosterone and aggression: A reanalysis of book, Starzyk, and Quinsey's (2001) study.* Aggression and Violent Behavior, 2005. **10**(2): p. 241-261.

217. Book, A.S., K.B. Starzyk, and V.L. Quinsey, *The relationship between testosterone and aggression: a meta-analysis.* Aggression and Violent Behavior, 2001. **6**(6): p. 579-599.

218. Cashdan, E., *Hormones and competitive aggression in women.* Aggressive Behavior, 2003. **29**(2): p. 107-115.

219. von der Pahlen, B., et al., *An exploratory study on self-evaluated aggression and androgens in women.* Aggressive Behavior, 2002. **28**(4): p. 273-280.

220. Manuck, S.B., et al., *A regulatory polymorphism of the monoamine oxidase-A gene may be associated with variability in aggression, impulsivity, and central nervous system serotonergic responsivity.* Psychiatry Research, 2000. **95**(1): p. 9-23.

221. Caspi, A., et al., *Role of genotype in the cycle of violence in maltreated children.* Science, 2002. **297**(5582): p. 851-854.

222. LaGrange, T.C. and R.A. Silverman, *Low self-control and opportunity: Testing the general theory of crime as an explanation for gender differences in delinquency.* Criminology, 1999. **37**(1): p. 41-72.

223. Nishizawa, S., et al., *Differences between males and females in rates of serotonin synthesis in human brain.* Proceedings of the National Academy of Sciences of the United States of America, 1997. **94**(10): p. 5308-5313.

224. Gur, R.C., et al., *Sex differences in temporo-limbic and frontal brain volumes of healthy adults.* Cereb Cortex, 2002. **12**(9): p. 998-1003.

225. Andersen, K., et al., *Gender differences in the incidence of AD and vascular dementia: The EURODEM Studies. EURODEM Incidence Research Group.* Neurology, 1999. **53**(9): p. 1992-7.

226. Gao, S., et al., *The relationships between age, sex, and the incidence of dementia and Alzheimer disease: a meta-analysis.* Arch Gen Psychiatry, 1998. **55**(9): p. 809-15.

227. Becker, J.B., *Sex differences in the brain : from genes to behavior*. 2008, Oxford University Press: Oxford ; New York. p. 442-445.

228. Kehoe, P., et al., *A full genome scan for late onset Alzheimer's disease.* Hum Mol Genet, 1999. **8**(2): p. 237-45.

229. Corder, E.H., et al., *Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families.* Science, 1993. **261**(5123): p. 921-3.

230. Johnson, J.K., et al., *Initiation and propagation stages of beta-amyloid are associated with distinctive apolipoprotein E, age, and gender profiles.* Brain Res, 1998. **798**(1-2): p. 18-24.

231. Callen, D.J., et al., *The influence of sex on limbic volume and perfusion in AD.* Neurobiol Aging, 2004. **25**(6): p. 761-70.

232. de Lau, L.M., et al., *Incidence of parkinsonism and Parkinson disease in a general population: the Rotterdam Study.* Neurology, 2004. **63**(7): p. 1240-4.

233. Van Den Eeden, S.K., et al., *Incidence of Parkinson's disease: variation by age, gender, and race/ethnicity.* Am J Epidemiol, 2003. **157**(11): p. 1015-22.

234. Twelves, D., K.S. Perkins, and C. Counsell, *Systematic review of incidence studies of Parkinson's disease.* Mov Disord, 2003. **18**(1): p. 19-31.

235. Baba, Y., et al., *Gender and the Parkinson's disease phenotype.* J Neurol, 2005. **252**(10): p. 1201-5.

236. Fernandez, H.H., et al., *Gender differences in the frequency and treatment of behavior problems in Parkinson's disease. SAGE Study Group. Systematic Assessment and Geriatric drug use via Epidemiology.* Mov Disord, 2000. **15**(3): p. 490-6.

237. Rojo, A., et al., *Depression in Parkinson's disease: clinical correlates and outcome.* Parkinsonism Relat Disord, 2003. **10**(1): p. 23-8.

238. Becker, J.B., *Sex differences in the brain : from genes to behavior*. 2008, Oxford University Press: Oxford ; New York. p. 458.

239. Foltynie, T., et al., *The BDNF Val66Met polymorphism has a gender specific influence on planning ability in Parkinson's disease.* J Neurol, 2005. **252**(7): p. 833-8.

240. Benedetti, M.D., et al., *Hysterectomy, menopause, and estrogen use preceding Parkinson's disease: an exploratory case-control study.* Mov Disord, 2001. **16**(5): p. 830-7.

241. Martignoni, E., et al., *Parkinson's disease and reproductive life events.* Neurol Sci, 2002. **23 Suppl 2**: p. S85-6.

242. Ragonese, P., et al., *Risk of Parkinson disease in women: effect of reproductive characteristics.* Neurology, 2004. **62**(11): p. 2010-4.

243. Maraganore, D.M., et al., *High-resolution whole-genome association study of Parkinson disease.* Am J Hum Genet, 2005. **77**(5): p. 685-93.

244. Pankratz, N., et al., *Genome-wide linkage analysis and evidence of gene-by-gene interactions in a sample of 362 multiplex Parkinson disease families.* Hum Mol Genet, 2003. **12**(20): p. 2599-608.

245. Hong, C.J., et al., *Brain-derived neurotrophic factor (BDNF) Val66Met polymorphisms in Parkinson's disease and age of onset.* Neurosci Lett, 2003. **353**(1): p. 75-7.

246. Di Monte, D.A., M. Lavasani, and A.B. Manning-Bog, *Environmental factors in Parkinson's disease.* Neurotoxicology, 2002. **23**(4-5): p. 487-502.

247. Modahl, C., et al., *Plasma oxytocin levels in autistic children.* Biol Psychiatry, 1998. **43**(4): p. 270-7.

248. De Vries, G.J., et al., *Sex differences in the effects of testosterone and its metabolites on vasopressin messenger RNA levels in the bed nucleus of the stria terminalis of rats.* J Neurosci, 1994. **14**(3 Pt 2): p. 1789-94.

249. Wang, Z., N.A. Bullock, and G.J. De Vries, *Sexual differentiation of vasopressin projections of the bed nucleus of the stria terminals and medial amygdaloid nucleus in rats.* Endocrinology, 1993. **132**(6): p. 2299-306.

250. Wu, S., et al., *Positive association of the oxytocin receptor gene (OXTR) with autism in the Chinese Han population.* Biol Psychiatry, 2005. **58**(1): p. 74-7.

251. Jacob, S., et al., *Association of the oxytocin receptor gene (OXTR) in Caucasian children and adolescents with autism.* Neurosci Lett, 2007. **417**(1): p. 6-9.

252. Yirmiya, N., et al., *Association between the arginine vasopressin 1a receptor (AVPR1a) gene and autism in a family-based study: mediation by socialization skills.* Mol Psychiatry, 2006. **11**(5): p. 488-94.

253. Wassink, T.H., et al., *Examination of AVPR1a as an autism susceptibility gene.* Mol Psychiatry, 2004. **9**(10): p. 968-72.

254. van Rijn, S., et al., *X Chromosomal effects on social cognitive processing and emotion regulation: A study with Klinefelter men (47,XXY).* Schizophr Res, 2006. **84**(2-3): p. 194-203.

255. Kosfeld, M., et al., *Oxytocin increases trust in humans.* Nature, 2005. **435**(7042): p. 673-6.

256. Kirsch, P., et al., *Oxytocin modulates neural circuitry for social cognition and fear in humans.* J Neurosci, 2005. **25**(49): p. 11489-93.

257. Heinrichs, M., et al., *Social support and oxytocin interact to suppress cortisol and subjective responses to psychosocial stress.* Biol Psychiatry, 2003. **54**(12): p. 1389-98.

258. Brady, K.T. and C.L. Randall, *Gender differences in substance use disorders.* Psychiatr Clin North Am, 1999. **22**(2): p. 241-52.

259. Kosten, T.R., B.J. Rounsaville, and H.D. Kleber, *Ethnic and gender differences among opiate addicts.* Int J Addict, 1985. **20**(8): p. 1143-62.

260. White, F.J., *A behavioral/systems approach to the neuroscience of drug addiction.* J Neurosci, 2002. **22**(9): p. 3303-5.

261. Justice, A.J. and H. De Wit, *Acute effects of d-amphetamine during the early and late follicular phases of the menstrual cycle in women.* Pharmacol Biochem Behav, 2000. **66**(3): p. 509-15.

262. Justice, A.J. and H. de Wit, *Acute effects of d-amphetamine during the follicular and luteal phases of the menstrual cycle in women.* Psychopharmacology (Berl), 1999. **145**(1): p. 67-75.

263. Robbins, S.J., et al., *Comparing levels of cocaine cue reactivity in male and female outpatients.* Drug Alcohol Depend, 1999. **53**(3): p. 223-30.

264. Kessler, R.C., et al., *Lifetime and 12-month prevalence of DSM-III-R psychiatric disorders in the United States. Results from the National Comorbidity Survey.* Arch Gen Psychiatry, 1994. **51**(1): p. 8-19.

265. Kennett, G.A., et al., *Female rats are more vulnerable than males in an animal model of depression: the possible role of serotonin.* Brain Res, 1986. **382**(2): p. 416-21.

266. Colangelo, L.A., et al., *Total testosterone, androgen receptor polymorphism, and depressive symptoms in young black and white men: the CARDIA Male Hormone Study.* Psychoneuroendocrinology, 2007. **32**(8-10): p. 951-8.

267. Seidman, S.N., et al., *Testosterone level, androgen receptor polymorphism, and depressive symptoms in middle-aged men.* Biol Psychiatry, 2001. **50**(5): p. 371-6.

268. Kendler, K.S., et al., *A longitudinal twin study of 1-year prevalence of major depression in women.* Arch Gen Psychiatry, 1993. **50**(11): p. 843-52.

269. Caspi, A., et al., *Influence of life stress on depression: moderation by a polymorphism in the 5-HTT gene.* Science, 2003. **301**(5631): p. 386-9.

270. Grigoriadis, S. and G.E. Robinson, *Gender issues in depression.* Ann Clin Psychiatry, 2007. **19**(4): p. 247-55.

271. Kessler, R., E. Frank, and (ed), *Gender differences in major depression. Epidemiological findings.*, in *Gender and its Effects on Psychopathology*. 2000, American Psychiatric Press: Washington. p. 61-84.

272. Seeman, M.V., *Psychopathology in women and men: focus on female hormones.* Am J Psychiatry, 1997. **154**(12): p. 1641-7.

273. Breslau, N., L. Schultz, and E. Peterson, *Sex differences in depression: a role for preexisting anxiety.* Psychiatry Res, 1995. **58**(1): p. 1-12.

274. Altemus, M., *Sex differences in depression and anxiety disorders: potential biological determinants.* Horm Behav, 2006. **50**(4): p. 534-8.

275. Harrison, P.J. and E.M. Tunbridge, *Catechol-O-methyltransferase (COMT): a gene contributing to sex differences in brain function, and to sexual dimorphism in the predisposition to psychiatric disorders.* Neuropsychopharmacology, 2008. **33**(13): p. 3037-45.

276. Barr, C.S., et al., *Sexual dichotomy of an interaction between early adversity and the serotonin transporter gene promoter variant in rhesus macaques.* Proc Natl Acad Sci U S A, 2004. **101**(33): p. 12358-63.

277. Jablensky, A., *Epidemiology of schizophrenia: the global burden of disease and disability.* Eur Arch Psychiatry Clin Neurosci, 2000. **250**(6): p. 274-85.

278. Preston, N.J., et al., *Gender differences in premorbid adjustment of patients with first episode psychosis.* Schizophr Res, 2002. **55**(3): p. 285-90.

279. Goldstein, J.M., *Sex, hormones and affective arousal circuitry dysfunction in schizophrenia.* Horm Behav, 2006. **50**(4): p. 612-22.

280. Di Paolo, T., *Modulation of brain dopamine transmission by sex steroids.* Rev Neurosci, 1994. **5**(1): p. 27-41.

281. Kulkarni, J., et al., *A clinical trial of the effects of estrogen in acutely psychotic women.* Schizophr Res, 1996. **20**(3): p. 247-52.

282. Feng, J., et al., *Evidence for X-chromosomal schizophrenia associated with microRNA alterations.* PLoS One, 2009. **4**(7): p. e6121.

283. Goldstein, J.M., et al., *Genetic heterogeneity may in part explain sex differences in the familial risk for schizophrenia.* Biol Psychiatry, 1995. **38**(12): p. 808-13.

284. Morris, A.G., et al., *CAG repeat expansions and schizophrenia: association with disease in females and with early age-at-onset.* Hum Mol Genet, 1995. **4**(10): p. 1957-61.

285. Munro, C.A., et al., *Sex differences in striatal dopamine release in healthy adults.* Biol Psychiatry, 2006. **59**(10): p. 966-74.

Chapter 2

The impact of sex chromosome complement on sex differences in adult mouse striatum

Abstract

The biological basis of sex differences in striatum and the vulnerability for many neurological disorders involving this brain region have been the subject of recent studies. Many of these differences have been attributed to the effect of sex steroid hormones. Evidence accumulated more recently, however, suggests that hormones are not the sole determinants and that perhaps the complement of sex chromosomes (XX vs. XY) is involved in the sexual differentiation of this brain region. To test this, we used the Four Core Genotypes (FCG) mouse model in which the genetic sex of the cells is unlinked from the gonadal sex (ovaries or testes). We found a number of genes that were differentially expressed between XX and XY animals due to sex chromosome complement. *Frmpd4*, an X-linked gene, was expressed at significantly higher levels in XY than in XX animals, irrespective of gonadal sex and the type of hormone treatment used. Using a bisulfite sequencing method, we found that there was an increase in mean DNA methylation levels of four CpG sites within the promoter region of *Frmpd4* in the striatum of XX relative to XY female mice of the Four Core Genotypes model. *Frmpd4* is involved in dendritic spine formation and clear sex differences in dendritic spine density have been identified in several brain regions including the striatum. Sex chromosome regulation of striatal gene expression has particular implications for neurologic disorders that affect the striatum, such as Parkinson's disease and addiction.

Key words: sex, sex chromosome complement, striatum, Frmpd4

Introduction

Sex differences in nigrostriatal function have been extensively documented. These include the sex differences in the expression of behavioral sensitization in response to striatal stimulation (female>male), the release of dopamine/DA (female>male), D1 dopamine receptor levels (male>female), and dopamine transporter mRNA density (female>male) [1-4]. Some of these differences are induced by sex differences in the circulating levels of gonadal steroids. For example, estrogen causes an increase in amphetamine-induced dopamine (DA) release and downregulates DA D2 receptors.

However, some of the sex differences in the striatum may be induced by direct genetic effects and/or sex chromosome gene expression [5-13].Our group has previously demonstrated that the Y chromosome-linked, male-determining gene *Sry* is specifically expressed in the tyrosine hydroxylase (TH)-expressing neurons of the substantia nigra (SN), a brain region that sends dopamine output to the striatum, in adult male rodents [14]. *Sry* knockdown in the male SN results in deficits in motor performance, marked depletions in striatal dopamine levels, and a concomitant reduction in nigrostriatal TH in these animals. In another independent study, Chen et al. examined for sex chromosome effects on expression of three mRNAs in the striatum and nucleus accumbens of adult mice of the Four Core Genotypes model. They found that XX mice had higher expression, relative to XY mice of the same gonadal sex, of two of these genes (prodynorphin and the substance P precursor Tac1) in specific striatal regions [15]. Altogether, these data suggest that sex differences in nigrostriatal function are attributable to a mixture of sex-specific effects of gonadal hormones and direct effects of sex-linked genes.

The research presented here addresses the contribution of sex chromosome genes to the global transcriptional networks that dictate the sex-differential expression of genes in striatum. Sex chromosome effects are tested by using the Four Core Genotypes (FCG) mouse model in which the genetic sex of the cells is separate from the gonadal sex (ovaries or testes). This model allows the independent assessment of the functional significance of sex chromosome genes versus that of gonadal sex and/or presence of *Sry*. Comparison of XX and XY mice with the same gonadal sex allows one to determine whether the sex differences in brain function and behavior are caused by the complement of sex chromosomes within cells [16, 17].

Our data suggested the existence of sexual dimorphism in the expression of striatal genes caused by the difference in complement of sex chromosomes between males and females. For example our results indicated that *Frmpd4,* a gene that is involved in regulation of dendritic spine formation and in metabotropic glutamate receptor signaling [18, 19], was highly sexually dimorphic in striatum and its sex-specific expression was attributed to the sex chromosome effects.

Materials and methods

Animals

All experiments were conducted using procedures approved by the UCLA Chancellor's Animal Care Committee. Mice were kept at a 12-h light/12-h dark cycle with food and water available *ad libitum*. The FCG used in these experiments were of C57BL/6J origin. In FCG mice, the testis-determining gene *Sry* is deleted from the Y chromosome and inserted back onto an autosome as a transgene. *Sry* deletion only removes a 12 kb region that is responsible for testis determination. XY mice without the autosomal *Sry* (XY^- mice) are gonadally female and in some instances referred to as XYF. On the other hand, mice with the autosomal *Sry* [XY^-(*Sry*+) animals or XYM] develop as gonadal males and have testes [20]. XY^-(*Sry*+) mice are fully fertile similar to XY males. Crossing XY^-(*Sry*+) males with XX females produce offspring with the genotypes XX and XY^-(*Sry*+) (XXF and XYM), in addition to two non-parental genotypes, XX(*Sry*+) and XY^- (XXM and XYF).

On postnatal day 75 (PN75), Mice were gonadectomized and subcutaneously implanted with estradiol, testosterone, or vehicle pellets. Testosterone pellets were made by plugging one end of a Silastic tube (1.57 mm ID x 2.41 mm OD, Dow Corning) with 3 mm of Silastic medical grade adhesive (Dow Corning) and letting it cure overnight. Then 5 mm of packed powdered testosterone (4-androsten-17β-OL-3, Steraloids Inc.), and 3 mm of Silastic medical grade adhesive was used to plug the other end [21]. Estradiol pellets were made by plugging one end of a Silastic tube (1.98 mm ID x 3.18 mm OD, Dow Corning) with 3 mm of Silastic medical grade adhesive (Dow Corning) and letting it cure overnight. Powdered 17β-estradiol (1,3,5(10)-estratrien-3,17β-diol, Steraloids Inc.) was dissolved in sesame oil (Sigma) with a ratio of 50 μg

estradiol in 25 μL sesame oil. A hamilton syringe was used to carefully add 25 μL of prepared oil to the silicone tube and the tube was sealed with 3 mm Silastic medical grade adhesive [22]. Vehicle pellets were made by filling Silastic tube (1.57 mm ID x 2.41 mm OD, Dow Corning) with Silastic medical grade adhesive (Dow Corning) and letting it cure overnight. Pellets were cured overnight, trimmed, and rinsed with 70% ethanol before implantation. The striatum was rapidly dissected on PN300 and then frozen at -80°C until processed for RNA using Qiagen's RNeasy Mini Kit.

MouseRef-8 v1.1 Illumina BeadChips

All total RNA samples were quantified using a Ribogreen fluorescent assay and normalized to 10 ng/ul prior to amplification. Amplified and labeled cRNA was produced using the Illumina specific Ambion TotalPrep kit (Applied Biosystems Inc., Foster City, CA). First and second strand cDNA were produced using the Ambion kit and purified using a robotic assisted magnetic capture step. Biotinylated cRNA was produced from the cDNA template in a reverse transcription reaction. Typical yields were in excess of 1.5 ug. After a second Ribogreen quant and normalization step, amplified and labeled cRNA was hybridized overnight at 58°C to the expression arrays. To minimize array-to-array variability, a cRNA sample from each of the testosterone, estradiol and vehicle treated FCG groups was hybridized to each of the beadchips (n = 5/group) according to the manufacturer's protocol. The MouseRef-8 v1.1 beadchip contains over 24,000 well-annotated RefSeq transcripts and allows eight samples to be interrogated in parallel. Hybridization was followed by washing, blocking, staining and drying on the Little Dipper processor. Array chips were scanned on either the Beadarray reader or the iScan reader (Illumina, Inc., San Diego, CA).

Quantitative RT-PCR

Total RNA (0.5 µg) from FCG mice (6-8 in each group) was used to synthesize cDNA in a 20 µl reaction using the Tetro cDNA Synthesis Kit with a combination of random priming and oligo dT (Bioline, Tauton, MA). The reverse transcription was performed at 45ºC for 1 hr. cDNAs were subjected to PCR with primers specific to mouse *Frmpd4*: Fwd 5'-CCACACTGGAAGCCCTAGAA-3' and Rev 5'-CTAGCTCAGAACCCTCTGTCA-3'; mouse *Prps2*: Fwd 5'-TCGAGATCGGTGAAAGTGTG-3' and Rev 5'-CGTAGGGGAAGCAGGGTATC-3'; and mouse *Tmsb4x*: Fwd 5' GGCTGAGATCGAGAAATTCG-3' and Rev 5'-TGATCCAACCTCTTTGCATCT-3'. Primers were designed with Primer3 software (The Whitehead Institute, Boston, MA). The β2-microglobulin gene served as a template control. Primers specific to mouse β2-microglobulin included: Fwd: 5'-TGGTGCTTGTCTCACTGACC-3' and rev 5'-GTATGTTCGGCTTCCCATTC-3'**.** PCR conditions used for amplification were as follows: initial denaturation at 95ºC for 10 min, 40 cycles of denaturation at 95ºC for 15 s, annealing at 60ºC for 15 s, and elongation at 72ºC for 30 s, with a final extension at 72ºC for 5 min. Amplified products were separated by electrophoresis on a 1.5% agarose gel with ethidium bromide and visualized under UV light to confirm product size.

Methylation analysis

To determine the methylation status of four CpG sites in the promoter region of *Frmpd4*, a minimum of 1 µg of DNA from each of the Four Core Genotypes (n=3) was treated with sodium bisulfite to convert unmethylated cytosines to uracil using the CpGenome™ DNA Modification Kit (Millipore, Billerica, MA, USA). Bisulfite-modified genomic DNA (8 µl) was

used as a template in a PCR reaction to amplify the *Frmpd4* gene promoter region using the Kappa 2G Robust Hot Start Kit (Kappa Biosystem, Cape Town, SA) in a 20 µl reaction mix containing 0.2 mM of each dNTP, 0.5 µM of each primer, and 1U/reaction Hot Start polymerase (Invitrogen, Eugene, OR). Amplification conditions were as follow: 95°C for 3 min, 45 cycles of 95°C for 10 s, 64°C for 30 s, and 72°C for 10 s. The following sets of primers were used: (1) Fwd 5′-AGAAAGTGTTTTAGAAAGGAGAGTTTA-3′ type and rev 5′-AATCCTTCAAAAAATTCTACCATTCC-3. PCR products were purified using the MinElute PCR Purification Kit (Qiagen, Valencia, CA), following the given protocol. Amplicons were cloned into pCR2.1 (TOPO-TA-Kit, Invitrogen). *E. coli* (OneShotH TOP10 chemically competent cells, Invitrogen) was transformed according to the manufacturer's protocol. A PCR was performed using the Kappa 2G Robust Hot Start Kit (Kappa Biosystem, Cape Town, SA) to identify positive clones. Single clones were sequenced using T7 primers. The methylation intensity for each individual was calculated by dividing the number of methylated sites in all clones by the number of possible methylation sites. The methylation frequency at individual CpG sites and total mean methylation levels at all CpG sites were calculated.

Statistics

Statistical analysis, including three-way ANOVA and False discovery rate (FDR), was performed in R (http://www.r-project.org/) for the microarray data. The threshold of 10% FDR was applied to all statistical measurements. Results of the quantitative RT-PCR for FCG mice were analyzed by two-way ANOVA, with factors of sex (gonadal male vs. gonadal female) and sex chromosome complement (XX vs. XY). For comparison of XX versus XY in our methylation analysis, we used unpaired t-test.

Results

In order to define a set of genes influenced by organizational hormone effects, sex chromosome complement, or circulating gonadal hormones, we performed microarray transcription profiling on total RNA isolated from the striatum of XX, XX(*Sry*+), XY^- and XY^- (*Sry*+) mice (N=5 for each genotype). These mice were gonadectomized on postnatal day 75 (PN75) followed by an administration of estradiol (E_2), testosterone (T), or vehicle, which allowed us to determine the acute effects of circulating gonadal secretions on sex differences in striatum. Gene expression levels were compared using a three-way ANOVA using gonadal sex, sex chromosome complement, and hormone treatment as the three factors. Genes that passed an estimated false discovery rate (FDR) threshold of 10% were viewed as being expressed differentially for all statistical differences measured. Using these criteria, 16 and 5 genes displayed sexually dimorphic expression caused by differences in sex chromosome complement, or hormone treatment, respectively (Table 2-1).

To detect expression differences attributed to the organizational effects of hormones, we compared mice differing in gonadal sex but with the same complement of sex chromosomes within each hormone treatment group. We found no genes that were differentially expressed in XX(*Sry*+) versus XX in E_2-, T-, or vehicle-treated groups. Similarly, there were no significantly differentially expressed genes detected between XY^-(*Sry*+) males and XY^- females across all hormone-treated groups. Although this observation is surprising, this could be due to the fact that our study is underpowered to detect expression differences attributed to the organizational effects of hormones. Moreover, previous studies on the liver, adipose, muscle, and the whole brain from more than 360 XX and XY mice indicate that the majority of sex differences in gene expression

are modest, with more than 70% of genes displaying less than a 1.2-fold difference in RNA levels [23].

To determine the relative contributions of activational effects of hormones, each genotype group was compared across different hormone treated groups. Five genes showed differential expression based on hormone treatment (Table 2-2).

To examine the effects of sex chromosome complement, we compared vehicle-treated XY and XX animals that had the same gonadal type. We found 16 genes to be differentially expressed between XY$^-$(*Sry*+) and XX(*Sry*+). As these mice are similar in their gonadal type but differ in their sex chromosome complement, this difference in gene expression can be attributed to the difference in sex chromosome composition. These genes were also different between vehicle-treated XY$^-$ and XX females. Six of these genes exhibited higher expression levels in XX as compared to XY and 10 displayed higher expression in XY relative to XX (Table 2-3). In general, genes that were differentially expressed between XY and XX mice overlapped completely across all hormone treatment groups (Table 2-3, Table 2-4, and Table 2-5).

Using quantitative RT-PCR performed on an independent sample set from vehicle-treated animals, we confirmed the observed XY versus XX expression differences in *Tmsb4x, Prsp2,* and *Frmpd4*. All three genes were upregulated in XY relative to the XX, regardless of the gonadal phenotype (Fig. 2-1). Interestingly, *Frmpd4*, *Prps2*, *Tlr7*, and *Tmsb4x* are all found to be located on the X chromosome, and given the fact that these genes demonstrate higher expression levels in XY relative to XX animals, it is likely that they represent an imprinted gene cluster.

Frmpd4 is differentially methylated between XX and XY

Among the list of genes with higher expression in XY versus XX mice, *Frmpd4* was of particular interest to us because this gene encodes a novel PSD-95-interacting protein with an

essential role in dendritic spine formation and excitatory synaptic transmission [24]. Recent reports suggest that dendritic spine density is sexually dimorphic in the striatum and that changes in dendritic spine density have important implications for striatal neuronal function.

This gene was among the top most differentially expressed genes between XX and XY mice in our dataset across all hormone-treated groups (Table 2-3 and Table 2-4, and Table 2-5) and its higher expression in XY relative to XX mice can potentially be explained by two mechanisms: 1) Y gene dosage; and 2) sex-specific imprinting of X chromosome genes.

In order to examine the contribution of sex-specific imprinting to the sex differences in *Frmpd4* expression and test out the possibility that the expression of this gene is regulated by epigenetic modifications, we examined and compared site-specific methylation of four CpG dinucleotides within the promoter region of *Frmpd4* in XX and XY animals of the FCG model. Sequence analysis (RefSeq Accession: NM_001033330) revealed that the *Frmpd4* promoter does not have typical CpG islands, but there are four CpG dinucleotides that are located near the transcription start site as shown in Fig. 2-2A. Our results indicated that there was a significant increase in mean DNA methylation levels of these four CpG sites in the XX relative to XY animals (Fig. 2-2D). With regards to individual CpG sites, methylation differences were clearer in female mice. In XYF, CpG 2 and 4 were hypomethylated relative to XXF. CpG 1 (67% methylated) was also hypomethylated in XY cells, albeit to a lesser extent (Fig. 2-2C).

Altogether, the observed differences in methylation levels between XY and XX animals in the *Frmpd4* regulatory region might potentially contribute to the observed differential expression.

Discussion

Many sexually dimorphic phenotypes are influenced by modest gene expression differences between males and females. Most of these differences have been attributed to discrete sex hormones produced by the two sexes, but some can also be mediated by genetic factors. Here, we try to uncover whether some of the sex differences in striatal gene expression in adult FCG mice are caused by the differences in sex chromosome complement, organizational effects of sex steroids, activational mechanisms, or by an interaction of all three variables.

Altogether, we were able to show that hormones are not the only determinants of brain sexual differentiation and sex chromosomes contribute to brain sex differences. This study allowed for the identification of several novel striatal genes whose sex-differential expression was dependent on the sex chromosome complement rather than gonadal hormones. *Frmpd4*, *Prps2*, *Tlr7*, and *Tmsb4x* were all found to be located on the X chromosome near the mouse pseudoautosomal region and in close proximity to a gene called *Msl3*, which is known to act within the dosage compensation complex in Drosophila [25]. Higher expression of these genes in XY relative to XX mice can potentially be explained by two mechanisms: 1) Y gene dosage; 2) sex-specific imprinting of X chromosome genes that could result in differential gene expression from the active paternal X versus the maternal X. However, given the fact that all these genes demonstrate higher expression levels in XY relative to XX animals, it is likely that they represent an imprinted gene cluster. Parental imprinting has been implicated in social behaviors, autism, parenting, aggression, nociception, and habit formation. Careful examination of the brain function of these X-linked genes and determining whether they are imprinted might give us important clues in understating brain sexual organization and interpreting sex differences in health and disease.

Among the list of genes with higher expression in XX versus XY mice, *Frmpd4* was of particular interest to us. Recently, it has been shown that *Frmpd4* binds group I metabotropic glutamate receptors (mGluRs) including mGluR1 and mGluR5, which are G protein-coupled receptors (GPCRs), involved in regulation of neuronal plasticity. These receptors are expressed at excitatory synapses in the brain and play an important role in inflammatory pain. Proline-directed kinases that phosphorylate the group I mGluRs at the binding site for the adaptor protein, Homer, facilitate mGluR–Homer binding and negatively regulate mGluR1 signaling. Interestingly, genetic disruption of Frmpd4 in mice inhibits the dynamic phosphorylation of mGluR5, resulting in Frmpd4−/− mice experiencing a persistent inflammatory pain that is caused by elevated mGluR signaling [24].

Frmpd4 is also a PSD-95-interacting protein. PSD-95, a postsynaptic density protein, has shown to be important in regulation of excitatory synapses and spine morphogenesis. In dendrites, *Frmpd4* is detected in PSD-95 clusters at postsynaptic sites and has been strongly implicated in regulation of dendritic spine density. Knockdown and dominant-negative inhibition of *Frmpd4* in cultured hippocampal neurons significantly reduces dendritic spine density, the number of excitatory synapses, and more importantly, excitatory synaptic transmission. Moreover, *Frmpd4* overexpression induces an increase in spine density, suggesting that Frmpd4 is involved in dendritic spine morphogenesis [24]. Changes in dendritic spine density have important implications for striatal neuronal function. For example, animals chronically subjected to psychostimulants exhibit an increase in their MSN spine density [26]. Recent reports suggest that dendritic spine density is sexually dimorphic in several brain regions. For example, female rats have been shown to have a higher density of large spines on medium spiny neurons in different subregions of nucleus accumbens (NAc), a region important in the neural circuitry of

reward and addiction. Synapses on these large spines are of an excitatory nature suggesting that females contain greater glutamatergic and dopaminergic input onto the dendrites of NAc. This has important implications for interpreting sex differences in addiction [27].

Progress in understanding the sexually dimorphic expression in the striatum, generally, and of *Frmpd4*, specifically, may also have important implications in Parkinson's disease. Parkinson's shows strong sex biases in prevalence (higher in males) and age-of-onset (earlier in males) [28]. Additionally, the medium spiny neurons (MSN) in the striatum of Parkinson's disease patients lose their dendritic spines [29, 30]. Therefore, these results suggest that *Frmpd4* could be involved in neurodegenerative diseases such as Parkinson's disease where their gender bias is not fully accounted for by effects of gonadal steroids.

The underlying factors leading to the sex chromosome-dependent expression of *Frmpd4* have yet to be elucidated. Interestingly, in agreement with our expression data, we observed higher methylation levels in XX relative to XY mice in the *Frmpd4* regulatory region suggesting that changes in DNA methylation might be linked to the sex-chromosome dependent differences in gene expression.

Our data also corroborated previous observations regarding the sex-specific differences in expression of X chromosome-inactivation escapees. *Kdm6a,* which encodes the histone H3K27me3 demethylase UTX; and *Eif2s3x,* the eukaryotic translation initiation factor 2, were found to be more highly expressed in XX than in XY mice. *Xist*, the major gene involved in the X-inactivation process was also higher in females than males. Since previous studies has been mostly performed on gonadally intact mice, our data extend these results by demonstrating that

the XX>XY expression of the X escapees is not caused by gonadal hormones but could rather be attributed to sex chromosome effects.

Altogether, our data suggests that the sex chromosomes carry genes that could influence the brain. These genetic effects independently or in concert with gonadal secretions can play important roles in brain function. Understanding the contribution of these different mechanisms to brain sexual differentiation can give us further insight into the physiology of the sexes and the differential vulnerability to and expression of neurodevelopmental disorders in males and females.

Acknowledgements

This work has been funded in part by the National Institutes of Health (NIH).

Figure 2-1. Quantitative RT-PCR of *Tmsb4x*, *Prps2* and *Frmpd4* was performed on an independent sample set. Gene expression comparison of *Tmsb4x*, *Prps2* and *Frmpd4* in striata microdissected from vehicle-treated gonadectomized (GDX) FCG mice is shown below. * p < 0.05; ** p< 0.01; *** p<0.001

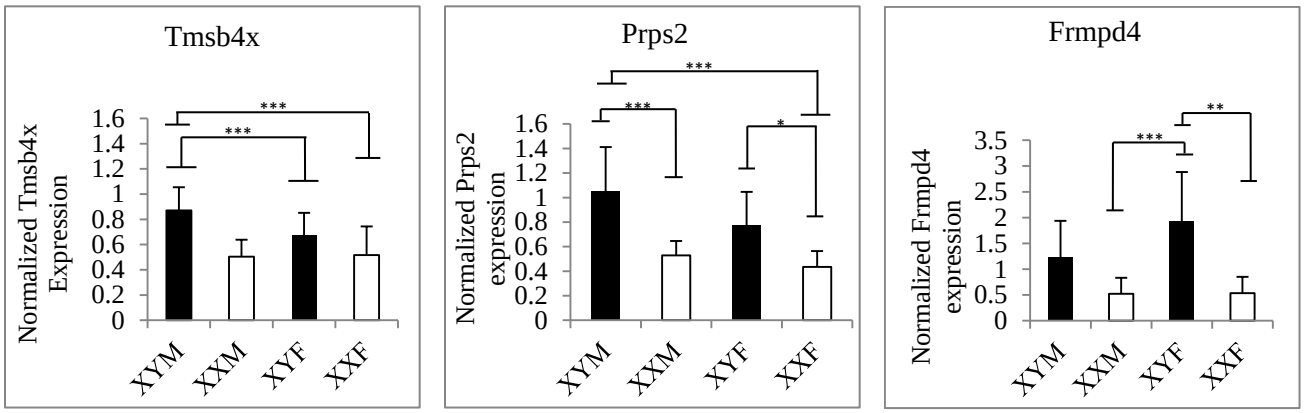


Figure 2-2: Methylation analysis of the four CpG sites within the *Frmpd4* gene promoter. **A.** Structure of the Frmpd4 gene promoter (above). The CpG sites sites are shown in red in the bottom. TSS: transcription start site. **B.** Sodium bisulfite DNA sequencing data of the four CpG sites located within the *Frmpd4* promoter in the FCG mice. Methylation is indicated as ● and unmethylated CpG as○. **C.** Degree (%) of methylation of the individual CpG sites is shown. **D.** Average methylation levels of the four CpG sites of XX versus. XY female and male animals. *: $P<0.05$ analyzed by unpaired t-test).

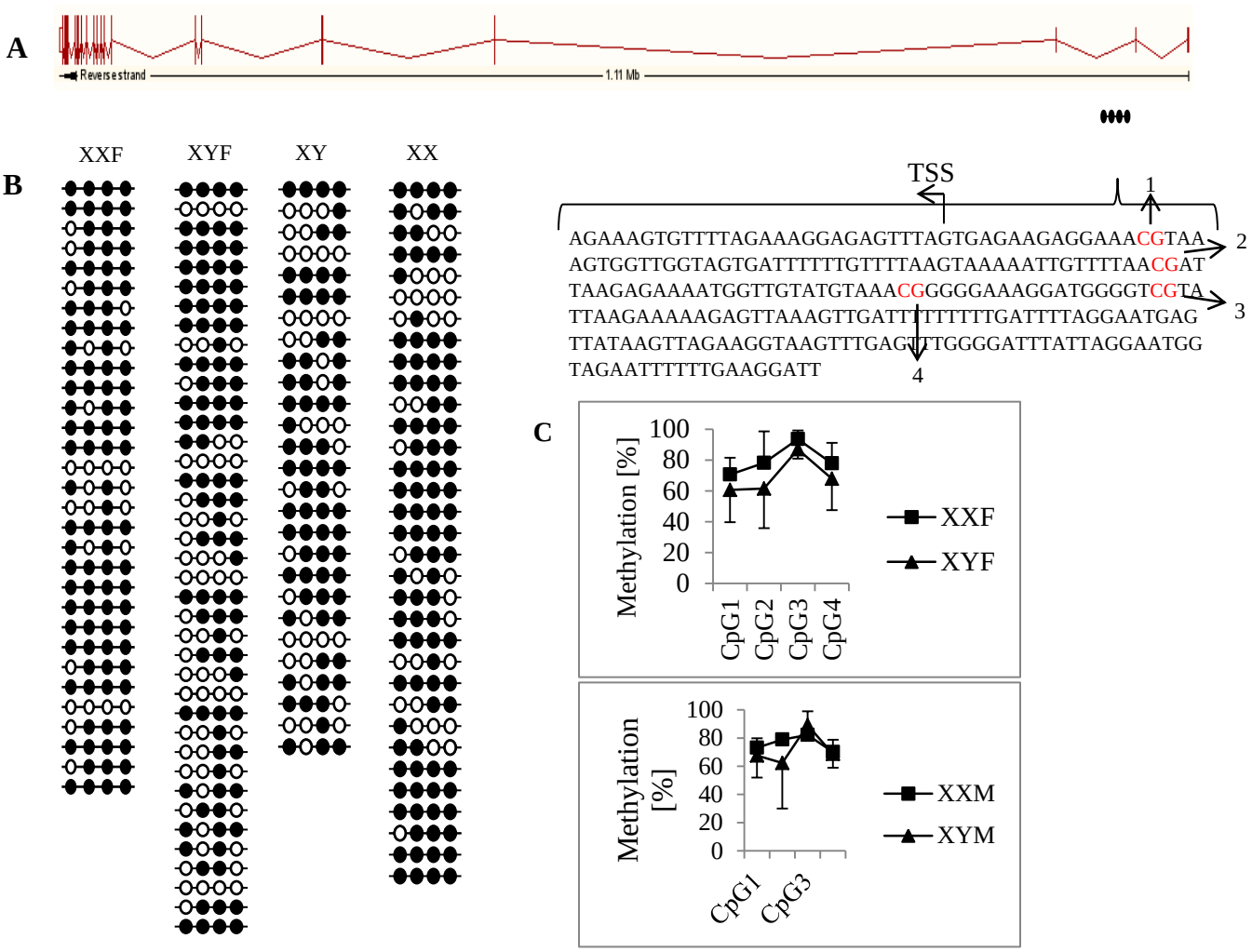

A
Reverse strand
1.11 Mb
B
XXF
XYF
XY
XX
TSS
1
2
3
4
AGAAAGTGTTTTAGAAAGGAGAGTTTAGTGAGAAGAGGAAACGTAA
AGTGGTTGGTAGTGATTTTTTGTTTTAAGTAAAAATTGTTTTAACGAT
TAAGAGAAAATGGTTGTATGTAAACGGGGGAAAGGATGGGGTCGTA
TTAAGAAAAAGAGTTAAAGTTGATTTTTTTTTGATTTTAGGAATGAG
TTATAAGTTAGAAGGTAAGTTTGAGTTTGGGGATTTATTAGGAATGG
TAGAATTTTTTGAAGGATT
C
Methylation [%]
100
80
60
40
20
0
CpG1
CpG2
CpG3
CpG4
XXF
XYF
Methylation [%]
100
80
60
40
20
0
CpG1
CpG3
XXM
XYM
D
*
Methylation [%]
100
80
60
40
20
0
XXF
XYF
*
Methylation [%]
100
80
60
40
20
0
XXM
XYM

Table 2-1: Number of genes that exhibit sex-specific expression due to chromosome composition, hormone treatment, or sex before and after FDR analysis.

Parameter	p value < 0.05	FDR < 0.1
Chromosome	997	16
Hormone	1637	5
Sex	654	0
Chromosome by Hormone **I**nteraction	607	0
Chromosome by Sex Interaction	630	0
Hormone by Sex Interaction	844	0
Chromosome by Hormone by Sex Interaction	602	0

Table 2-2: Top differentially expressed genes showing a main effect of hormone treatment. Numbers represent the mean of transcript abundance in **A.** vehicle; **B.** testosterone; **C.** estradiol treated groups. Statistical significance was measured by 3-way ANOVA. FDR <0.1.

A

Vehicle

GENE SYMBOL	MEAN XYSRY	MEAN XXSRY	MEAN XY	MEAN XX	p-value
HSD11B1	241.0398	279.5079	234.1572	220.566	9.995E-08
MSR2	204.5958	242.758	225.91	280.9172	2.057E-07
ITGAE	5.927443	3.367708	2.648538	-0.02024	2.2E-07
TGFBR1	1545.375	1527.412	1506.063	1544.697	1.518E-06
NT5E	363.1565	397.5364	414.3623	400.1786	1.773E-05
HSD11B1	452.6044	474.1924	377.3509	376.5305	2.626E-05

B

Testosterone

GENE SYMBOL	MEAN XYSRY	MEAN XXSRY	MEAN XY	MEAN XX	p-value
HSD11B1	172.1037	164.3242	179.8105	171.0901	9.995E-08
MSR2	208.4876	286.867	228.2258	267.9299	2.057E-07
ITGAE	13.30996	4.44767	4.918076	8.585337	2.2E-07
TGFBR1	1351.087	1593.731	1468.461	1505.588	1.518E-06
HMGCS1	8702.233	8383.113	7960.16	8128.98	1.712E-06
NT5E	546.0526	657.4839	502.1212	530.8252	1.773E-05

C

Estradiol

GENE SYMBOL	MEAN XYSRY	MEAN XXSRY	MEAN XY	MEAN XX	p-value
HSD11B1	147.5908	170.9341	146.3642	195.3741	9.995E-08
MSR2	377.2745	350.2699	317.6706	411.716	2.057E-07
ITGAE	22.76203	16.7857	21.84969	28.33771	2.2E-07
TGFBR1	1728.952	1680.446	1835.957	1894.621	1.518E-06
HMGCS1	8022.682	7579.615	6992.555	7045.232	1.712E-06
NT5E	513.4118	426.807	511.2127	645.8834	1.773E-05

Table 2-3: Differentially expressed genes between vehicle-treated XY vs. XX FCG mice.

Numbers represent the mean of transcript abundance in each group. Statistical significance was measured by 3-way ANOVA.

GENE SYMBOL	MEAN XYSRY	MEAN XXSRY	MEAN XY	MEAN XX	P VALUE	FDR
	XX > XY					
XIST	13.11	293.52	5.318288	303.9714	2.7E-29	3.47E-25
EIF2S3X	837.76	1370.89	900.9616	1188.765	2.33E-15	9.96E-12
UTX	524.12	767.72	536.702	679.8649	4.74E-09	1.01E-05
IGF2	1404.21	1462.05	1461.286	1956.521	6.71E-06	0.010143
CD59A	935.20	1244.99	833.2567	1121.563	8.66E-06	0.012363
2610029G23RIK	578.39	709.80	570.7856	692.6914	1.27E-05	0.01638
	XY > XX					
DDX3Y	1761.84	312.01	1773.745	233.7361	5.45E-23	4.67E-19
BTG4	205.08	109.40	198.8654	94.93179	5E-15	1.84E-11
TLR7	91.28	45.14	125.0604	57.3498	3.82E-14	1.23E-10
HCCS	201.39	101.50	229.2967	94.80827	1.43E-12	4.09E-09
FRMPD4	802.86	462.14	1041.789	448.1884	4.12E-11	1.06E-07
PRPS2	109.67	51.23	111.3489	46.99801	4.36E-08	7.46E-05
ARHGAP6	220.58	109.83	191.287	145.2454	3.03E-10	7.08E-07
2310047I15RIK	188.02	136.16	170.2452	133.7741	9.61E-06	0.012998
MSL31	39.78	19.55	50.06449	16.41456	4.9E-05	0.059944
TMSB4X	13870.38	11073.16	13129.63	10345.28	6.59E-05	0.077027

Table 2-4: Differentially expressed genes between testosterone-treated XY vs. XX FCG mice. Numbers represent the mean of transcript abundance in each group. Statistical significance was measured by 3-way ANOVA.

GENE SYMBOL	MEAN XYSRY	MEAN XXSRY	MEAN XY	MEAN XX	P VALUE	FDR
			XX > XY			
XIST	0.070689	260.5776	8.138605	299.7496	2.7E-29	3.47E-25
EIF2S3X	933.1397	1283.743	815.2775	1297.429	2.33E-15	9.96E-12
UTX	578.9929	805.1825	521.5548	757.8348	4.74E-09	1.01E-05
IGF2	1252.928	1668.986	1414.871	1568.829	6.71E-06	0.010143
CD59A	970.0247	1095.202	860.0644	1144.724	8.66E-06	0.012363
2610029G23RIK	587.0404	711.02	558.4246	705.088	1.27E-05	0.01638
			XY > XX			
DDX3Y	1861.075	300.0218	1723.118	322.5311	5.45E-23	4.67E-19
BTG4	189.3976	100.3931	202.356	105.4305	5E-15	1.84E-11
TLR7	100.833	59.71454	102.8226	48.93318	3.82E-14	1.23E-10
HCCS	217.5958	121.1932	215.134	108.0891	1.43E-12	4.09E-09
FRMPD4	824.3076	382.7411	793.4493	469.3775	4.12E-11	1.06E-07
PRPS2	72.0722	37.5721	74.9232	49.5321	4.36E-08	7.46E-05
ARHGAP6	239.8936	117.5847	248.3038	99.86112	3.03E-10	7.08E-07
2310047I15RIK	160.1685	130.2541	198.461	124.6927	9.61E-06	0.012998
MSL31	39.05105	18.47569	28.87467	21.39423	4.9E-05	0.059944
TMSB4X	13869.9	11941.87	15059.72	10728.8	6.59E-05	0.077027

Table 2-5: Differentially expressed genes between estradiol-treated XY vs. XX FCG mice.

Numbers represent the mean of transcript abundance in each group. Statistical significance was measured by 3-way ANOVA.

GENE SYMBOL	MEAN XYSRY	MEAN XXSRY	MEAN XY	MEAN XX	P VALUE	FDR
			XX > XY			
2610029G23RIK	3.90096344	295.266134	0.886765	318.1025	2.7E-29	3.47E-25
BTG4	15.4591396	233.218162	10.40998	283.0207	2.33E-15	9.96E-12
TLR7	1549.43199	1592.75731	1384.959	1830.305	4.74E-09	1.01E-05
HCCS	505.916304	764.294848	574.2699	700.8361	6.71E-06	0.010143
TMSB4X	776.317506	1180.64613	970.6548	1168.324	8.66E-06	0.012363
			XY > XX			
XIST	26.1619203	17.9478994	39.04162	15.4752	5.45E-23	4.67E-19
EIF2S3X	215.822787	96.9103056	227.6877	99.67674	5E-15	1.84E-11
UTX	144.180131	127.995508	169.7709	144.6368	3.82E-14	1.23E-10
IGF2	7.51192832	-2.1740539	4.842225	-2.1427	1.43E-12	4.09E-09
CD59A	34.437938	1.25530231	50.34116	2.129982	4.12E-11	1.06E-07
DDX3Y	114.698434	65.101656	146.3546	69.52217	4.36E-08	7.46E-05
FRMPD4	242.871366	118.712022	297.4133	135.1698	3.03E-10	7.08E-07
PRPS2	17.3984106	7.30711824	19.85071	-1.27256	9.61E-06	0.012998
ARHGAP6	1575.38248	274.128446	1955.437	309.3612	4.9E-05	0.059944
2310047I15RIK	343.75988	-9.7876158	308.2423	-3.4845	6.59E-05	0.077027
MSL31	76.6953182	43.5224455	107.4038	55.20163	5.45E-23	4.67E-19

References

1. Becker, J.B., *Gender differences in dopaminergic function in striatum and nucleus accumbens.* Pharmacol Biochem Behav, 1999. **64**(4): p. 803-12.
2. McArthur, S., E. McHale, and G.E. Gillies, *The size and distribution of midbrain dopaminergic populations are permanently altered by perinatal glucocorticoid exposure in a sex- region- and time-specific manner.* Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2007. **32**(7): p. 1462-76.
3. Walker, Q.D., et al., *Dopamine release and uptake are greater in female than male rat striatum as measured by fast cyclic voltammetry.* Neuroscience, 2000. **95**(4): p. 1061-70.
4. Ji, J. and D.E. Dluzen, *Sex differences in striatal dopaminergic function within heterozygous mutant dopamine transporter knock-out mice.* Journal of neural transmission, 2008. **115**(6): p. 809-17.
5. Chen, X., W. Grisham, and A.P. Arnold, *X chromosome number causes sex differences in gene expression in adult mouse striatum.* The European journal of neuroscience, 2009. **29**(4): p. 768-76.
6. Carrer, H.F. and M.J. Cambiasso, *Sexual differentiation of the brain: genes, estrogen, and neurotrophic factors.* Cellular and molecular neurobiology, 2002. **22**(5-6): p. 479-500.
7. Toran-Allerand, C.D., *Minireview: A plethora of estrogen receptors in the brain: where will it end?* Endocrinology, 2004. **145**(3): p. 1069-74.
8. Reisert, I. and C. Pilgrim, *Sexual differentiation of monoaminergic neurons--genetic or epigenetic?* Trends in neurosciences, 1991. **14**(10): p. 468-73.

9. Becker, J.B. and M.E. Beer, *The influence of estrogen on nigrostriatal dopamine activity: behavioral and neurochemical evidence for both pre- and postsynaptic components.* Behavioural brain research, 1986. **19**(1): p. 27-33.

10. Dluzen, D.E. and J.L. McDermott, *Developmental and genetic influences upon gender differences in methamphetamine-induced nigrostriatal dopaminergic neurotoxicity.* Annals of the New York Academy of Sciences, 2004. **1025**: p. 205-20.

11. Arnold, A.P. and P.S. Burgoyne, *Are XX and XY brain cells intrinsically different?* Trends in endocrinology and metabolism: TEM, 2004. **15**(1): p. 6-11.

12. Arnold, A.P., et al., *Minireview: Sex chromosomes and brain sexual differentiation.* Endocrinology, 2004. **145**(3): p. 1057-62.

13. Carruth, L.L., I. Reisert, and A.P. Arnold, *Sex chromosome genes directly affect brain sexual differentiation.* Nature neuroscience, 2002. **5**(10): p. 933-4.

14. Dewing, P., et al., *Direct regulation of adult brain function by the male-specific factor SRY.* Curr Biol, 2006. **16**(4): p. 415-20.

15. Chen, X., W. Grisham, and A.P. Arnold, *X chromosome number causes sex differences in gene expression in adult mouse striatum.* Eur J Neurosci, 2009. **29**(4): p. 768-76.

16. De Vries, G.J., et al., *A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits.* J Neurosci, 2002. **22**(20): p. 9005-14.

17. Lovell-Badge, R. and E. Robertson, *XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy.* Development, 1990. **109**(3): p. 635-46.

18. Lee, H.W., et al., *Preso, a novel PSD-95-interacting FERM and PDZ domain protein that regulates dendritic spine morphogenesis.* J Neurosci, 2008. **28**(53): p. 14546-56.

19. Hu, J.H., et al., *Preso1 dynamically regulates group I metabotropic glutamate receptors.* Nat Neurosci, 2012. **15**(6): p. 836-44.

20. Mahadevaiah, S.K., et al., *Mouse homologues of the human AZF candidate gene RBM are expressed in spermatogonia and spermatids, and map to a Y chromosome deletion interval associated with a high incidence of sperm abnormalities.* Hum Mol Genet, 1998. **7**(4): p. 715-27.

21. Smith E.R., D.D., Davidson J.M., *Hormone administration: peripheral and intracranial implants*. Methods in Psychobiology, ed. M. RD1977, New York: Academic Press.

22. Kudwa, A.E., et al., *Regulation of progestin receptors in medial amygdala: estradiol, phytoestrogens and sex.* Physiology & behavior, 2009. **97**(2): p. 146-50.

23. Yang, X., et al., *Tissue-specific expression and regulation of sexually dimorphic genes in mice.* Genome Res, 2006. **16**(8): p. 995-1004.

24. Lee, H.W., et al., *Preso, a novel PSD-95-interacting FERM and PDZ domain protein that regulates dendritic spine morphogenesis.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2008. **28**(53): p. 14546-56.

25. Lucchesi, J.C., *Dosage compensation in flies and worms: the ups and downs of X-chromosome regulation.* Current opinion in genetics & development, 1998. **8**(2): p. 179-84.

26. Li, Y., B. Kolb, and T.E. Robinson, *The location of persistent amphetamine-induced changes in the density of dendritic spines on medium spiny neurons in the nucleus accumbens and caudate-putamen.* Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology, 2003. **28**(6): p. 1082-5.

27. Forlano, P.M. and C.S. Woolley, *Quantitative analysis of pre- and postsynaptic sex differences in the nucleus accumbens.* The Journal of comparative neurology, 2010. **518**(8): p. 1330-48.

28. Ngun, T.C., et al., *The genetics of sex differences in brain and behavior.* Front Neuroendocrinol, 2011. **32**(2): p. 227-46.

29. Stephens, B., et al., *Evidence of a breakdown of corticostriatal connections in Parkinson's disease.* Neuroscience, 2005. **132**(3): p. 741-54.

30. Zaja-Milatovic, S., et al., *Dendritic degeneration in neostriatal medium spiny neurons in Parkinson disease.* Neurology, 2005. **64**(3): p. 545-7.

Chapter 3

Feminization of partner preference and brain gene expression in the Sex Chromosome Trisomy model, a novel mouse model of Klinefelter Syndrome

The work presented here is a collaborative effort between members of Dr. Arnold's group, myself, and Tuck Ngun, which consisted of me being the lead in performing all the gene expression analyses in the striatum (Figures 3-3 C and D; quantitative RT-PCR confirmation of feminized genes in Klinefelter male mice in the striatum in Figure 3-4; Figure 3-5B; Table 3-5; Table 3-7; Supplementary Table 3-1 striatum tab, Supplementary Table 3-2 striatum tab; and supplementary Table 3-3 striatum tab). Even though Tuck was the main person performing the behavioral assessments, I also contributed to the intellectual and performance aspects of the behavioral tests and in preparation of Figure 3-1, Figure 3-2, and Tables 3-1, 3-2, and 3-3.

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 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 (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 paper, 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 paper, 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 3-1A 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 just two generations. 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 paper) 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) and the striatum, two regions in the brain that are 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 and striatum 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 both brain regions. 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. The striatum was defined as the tissue between the external capsule and the anterior commissure, bounded laterally by the cortex and medially by 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 underwent partner preference testing. All animals were sexually naïve. For a schematic of the testing apparatus see Figure 3-1B. 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. Only data for male animals were analyzed. 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 one-tailed 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

Since we found modest sex differences in gene expression, we used a less stringent cut off than what was used in Chapter 2 to identify genes that were sexually dimorphic. In this study, 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 3-1. 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

To validate our experimental setup, we first 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 Mann-Whitney test; Table 3-2). The timeline for this study is presented in Figure 3-1C. The results of these tests are summarized in Table 3-2.

Partner preference in XXY and XX males

We 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 3-2).

We then analyzed time spent with each stimulus sex separately. Based on the higher rates of homosexual behavior seen in KS men, we 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. We 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 one-tailed Mann-Whitney test; Fig. 3-2A). 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 one-tailed Mann-Whitney test; Fig. 3-2B). 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 3-2). 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, we 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 3-3. 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 and striatum of XXY males

We then turned our 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 two regions of the brain that display sexual dimorphisms: the striatum and the BNST/POA. Genes that display basal sex differences in the two regions were first determined. 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 genes in the BNST and 364 genes in the striatum (Supp. Table 3-1), with 18 genes found to be sexually dimorphic in both regions.

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 are visualized in Fig. 3-3A and C. 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 in the BNST/POA, 270/364 in the striatum) so gene expression in XXYM in both brain regions is not generally feminized. However a minority of genes scored 70 or above (30 in the BNST/POA; 36 in the striatum). 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 whereas 24 out of 364 sexually dimorphic genes in the striatum qualified. The expression patterns of these feminized genes in XXYM, XYM, and XXF are visualized as heat maps in Fig. 3-3B and D. The proportion of feminized genes in the two regions are much higher than would be expected (null hypothesis: the only feminized genes will be X-inactivation escapees; $p<0.0001$ by the chi square test for both regions). 5 genes were common to the list of feminized genes in the two regions: *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 and striatum.

Differential expression of a number of feminized genes was verified using qRT-PCR. Those results are consistent with the microarray data (Fig. 3-4).

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

We were also interested in determining the effect of the additional X chromosome in XXYM on gene expression in the BNST/POA and striatum 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). In the BNST/POA, 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 3-2). In the striatum, there were 69 differentially expressed genes – 17 which were higher in XXYM and 52 which were lower (Supp. Table 3-2). 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. Only 7 genes were found to differentially expressed in both the BNST/POA and the striatum.

We 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. In the BNST/POA, 8 of the top 10 pathways affected by being XXYM were related to immune function (Table 3-4). In the striatum, although pathways affected by being XXYM segregated were more varied, immune function-related ones were still dominant as they made up 5 of the top 10 affected pathways (Table 3-5).

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. In the BNST/POA, 164 genes were differentially expressed between XXM and XYM (Supp. Table 3-3). In the striatum, 208 genes were different between XXM and XYM (Supp. Table 3-3). Next, we compared these lists to the ones generated by XXYM vs. XYM (Fig 3-5). We found that of the 190 genes different between XXYM and XYM in the BNST/POA, 170 were unique to that comparison (Fig 3-5A, yellow). In the striatum, 51 of the 70 genes were unique (Fig 3-5B, 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.

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. 20/170 genes in the BNST/POA are affected by being a 2X male (Table 3-6 and Fig 3-5A,

green). 19 of these genes were affected by being a 2X male (Table 3-7 and Fig 3-5B, green). In both regions of the brain, 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. The single exception to this is *Dnalc1* in the striatum, which is downregulated in XXYM vs. XYM but upregulated in XXM vs. XYM. *Dnalc1* codes for the light chain of axonemal dyneins and its expression is downregulated in a mouse model of multiple sclerosis [55]. 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, we used a tri-compartment apparatus that prevents physical contact between the test and stimulus animals. This allowed us 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, our 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 we 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, 56]. 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, 57]. 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 we 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.

We first tested if partner preference was feminized in XXYM compared to XYM. We 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, we 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.

In addition, there may have been differences in hormonal levels between XXYM and XYM prior to gonadectomy 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 we 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, we 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 [56]. 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 and striatum 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 [58, 59]. 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]. The striatum, the main region involved in dopaminergic function and reward, displays several key sex differences, many of which are caused by gonadal steroid hormones such as estrogen. For instance, dopamine biosynthesis [60], concentration [61, 62], degradation

[63], uptake [64, 65], and receptor density [66]; as well as variations in the concentration of basal extracellular striatal DA [67], amphetamine (AMPH)-stimulated DA release [67], and behaviors mediated by striatal DA are all dependent on estrogen [67]. Estrogen also works directly on the nucleus accumbens via a G-protein–coupled external membrane receptor, by increasing DA release and other DA-mediated behaviors [67]. Several studies have also highlighted the association between estrogen levels and dopamine transmission in humans [68]. Notably, however, there have also been observed sex differences in striatal DA transmission that appear to be caused independently of hormones. Recent studies have shown that *Sry* (the Y chromosome gene involved in male sex determination), is specifically expressed in the dopaminergic neurons of the substantia nigra in adult male rodents where it enhances striatum DA release and regulates sensorimotor functions of dopaminergic neurons [69]. Such data adds onto the increasing evidence that along with the effect of hormones, sex chromosome-linked genes are critical to sexually dimorphic neural and behavioral traits.

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. Although there was not much overlap between the two regions on the individual gene level, feminized genes in the BNST/POA and striatum show some overlap in molecular functions. Feminized genes in both regions are involved in apoptosis, regulation of cell cycle/proliferation, and neurodevelopment and function. *Snca* or *Park1*, was feminized in the striatum only (score of 88.9). It is well-known for its association with Parkinson's disease, which not only shows a male bias in incidence but also has gender differences in pathophysiology [70]. Women with Parkinson's more often present with

tremor, but so do KS men [71]. 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.

We were particularly interested in the question of whether any of these feminized genes played a role in the immune system. Emerging evidence indicates that there is crosstalk between the immune, nervous and endocrine systems (reviewed in [72]). 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 [73, 74]. 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 [72]. Many gender-biased neuropsychiatric traits and disorders such as addiction, autism, schizophrenia, and depression have also been linked to disruptions of the immune system [75-78].

Interestingly, the feminized genes in the BNST/POA was largely made up of those with known neurodevelopmental roles while genes that were feminized in the striatum were a mixture of neural development-related genes and those with immune functions. For instance, Spag9 (also known as JLP), is feminized in the BST/POA (feminization score of 100.2) and interacts with N-cadherin and links it to p38 MAPK signaling [79]. 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 [80]. An example of a neurodevelopment gene that is feminized in the striatum is *Pou3f4* (also called *Brn4*) (feminization score of 71.4). It is required for the differentiation of neural stem cells into neuronal cells and the subsequent maturation of these newborn neurons in the hippocampus [81]. *Pou3f4* can also induce neural stem cells to differentiate into dopaminergic neurons, which are crucial to striatal function [82]. *Ifna1* (feminization score of 98.5) is more highly expressed in XXYM and XXF compared to XYM. It codes for interferon-alpha which has been linked to the pathogenesis of systemic lupus erythematosus, a strongly female-biased autoimmune disease [83]. Its expression appears to be suppressed by estrogen [84]. In summary, these data suggest feminization of nervous system and immune genes may play a larger role in the striatum than in the BNST/POA in bringing about differences between XXYM and XYM.

Next, we sought to elucidate biological pathways that were different between XXYM and XYM. In both the BNST/POA and the striatum, immune function pathways were those that were most significantly affected by the XXY genotype. In the BNST/POA, 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. In the striatum, almost all the immune-related pathways affected by being XXY were involved in both innate and adaptive immunity. Once again, only a single pathway played a role exclusively in innate immunity. It is intriguing that pathways which play a role in both innate and adaptive immunity feature prominently in the datasets from both brain regions. PGE2 is able to take part in both types of 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 [72, 85]. 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.

We 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. We 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. In the BNST/POA, there are four such genes (*Cdc45l*, *Hsd3b2*, *Serpinh1*, and *Thoc3*) whereas there is only one gene in both categories in the striatum (*2310057J16Rik* or *Camsap3*). *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 our 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 [86]. 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 we are 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) [87]. Since then, other groups have also noted differences between 45,X^P and 45, X^M women on cognitive and physical phenotypes [88-90]. 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 [91]. A further complication is non-random X inactivation which happens in at least a subset of KS patients and may amplify these effects [92]. 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.

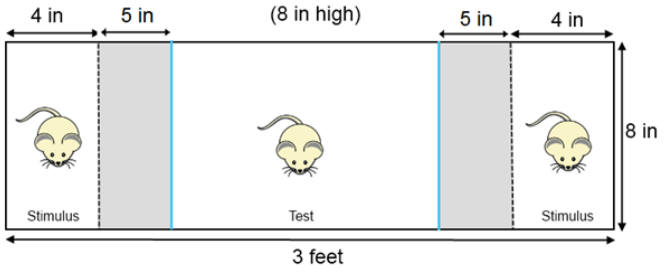
FIGURES AND TABLES

Figure 3-1: 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

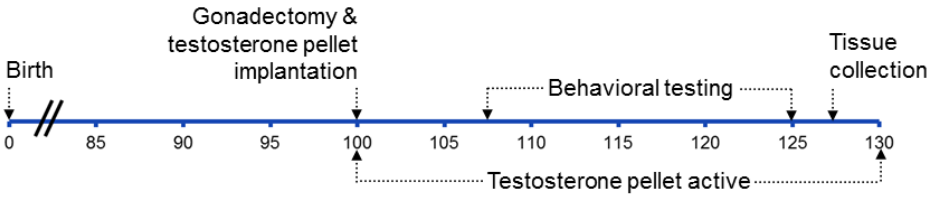


Figure 3-2: Time spent with the stimulus animal. The boxplot depicts time spent with the stimulus animal of each sex. Lines through the boxes indicate the median time in each group. Whiskers represent the limits of the upper and lower quartiles. **A.** XXY, but not XX, males spend significantly less 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.

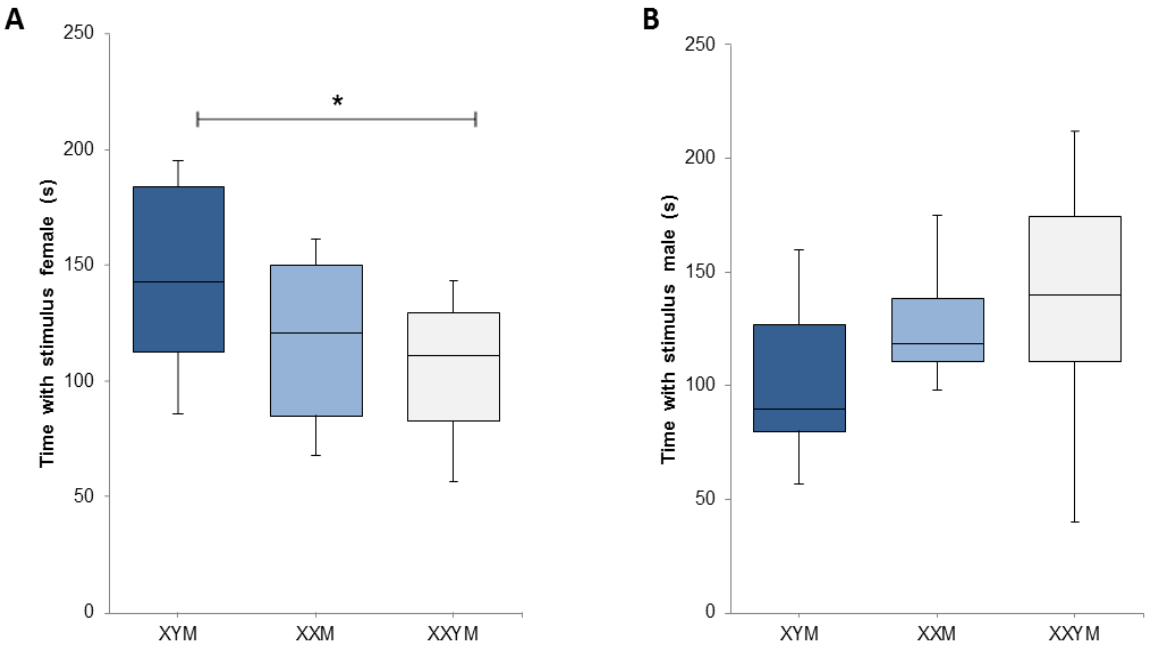


Fig. 3-3: Assessment and visualization of the degree of feminization of brain gene expression in XXYM. Mean XXYM expression of sexually dimorphic genes were assigned a feminization score and plotted on a continuum between average XYM and XXF expression in **A.** the BNST/POA, and **C.** the striatum. 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 in **B**. the BNST/POA and **D**. the striatum that survive Benjamini-Hochberg correction (FDR=10%) are visualized using a heat map.

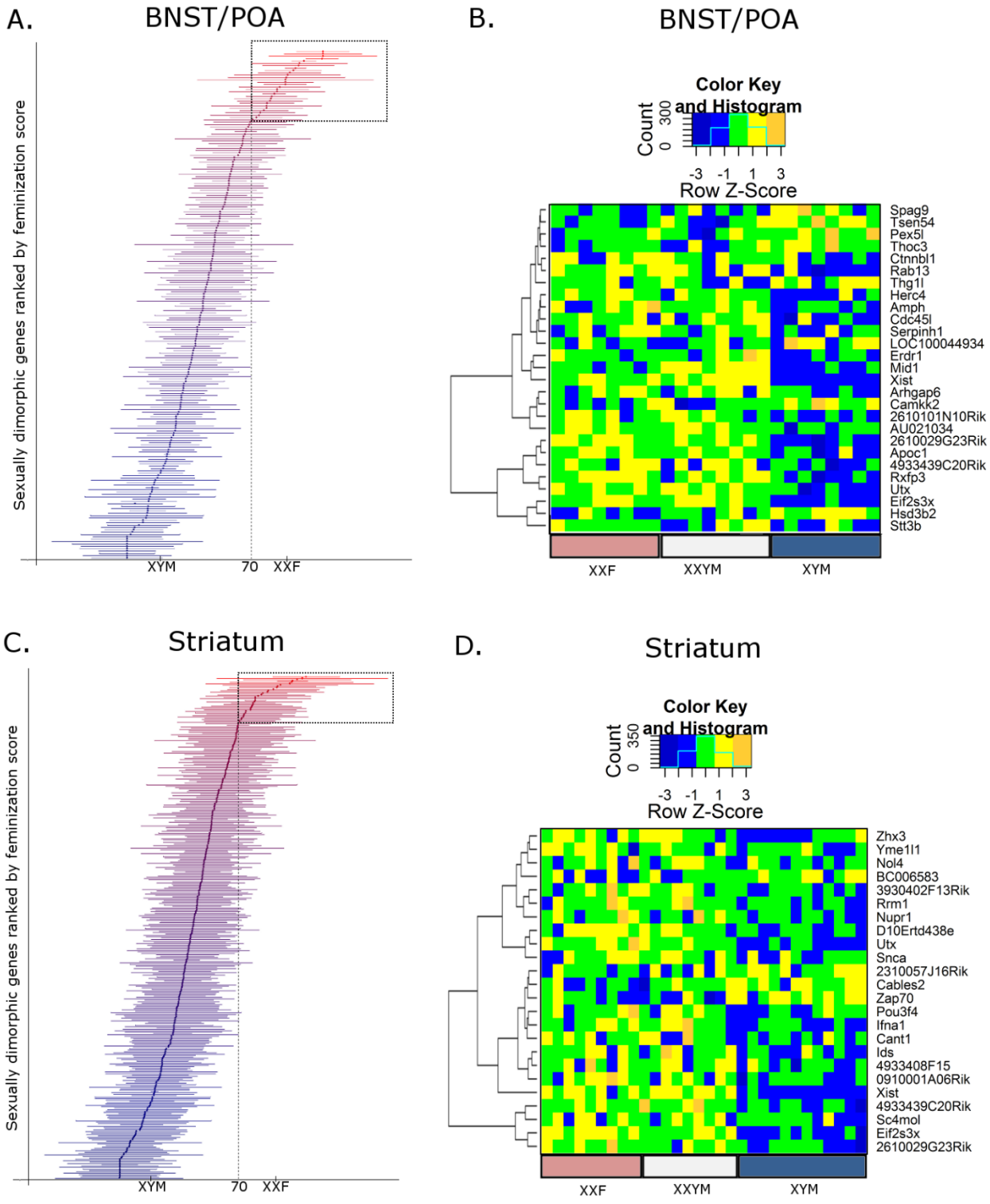

A.
BNST/POA
Sexually dimorphic genes ranked by feminization score
XYM
70
XXF
B.
BNST/POA
Color Key and Histogram
Count
0
300
-3
-1
1
3
Row Z-Score
Spag9
Tsen54
Pex5l
Thoc3
Ctnnbl1
Rab13
Thg1l
Herc4
Amph
Cdc45l
Serpinh1
LOC100044934
Erdr1
Mid1
Xist
Arhgap6
Camkk2
2610101N10Rik
AU021034
2610029G23Rik
Apoc1
4933439C20Rik
Rxfp3
Utx
Eif2s3x
Hsd3b2
Stt3b
XXF
XXYM
XYM
C.
Striatum
Sexually dimorphic genes ranked by feminization score
XYM
70
XXF
D.
Striatum
Color Key and Histogram
Count
0
350
-3
-1
1
3
Row Z-Score
Zhx3
Yme1l1
Nol4
BC006583
3930402F13Rik
Rrm1
Nupr1
D10Ertd438e
Utx
Snca
2310057J16Rik
Cables2
Zap70
Pou3f4
Ifna1
Cant1
Ids
4933408F15
0910001A06Rik
Xist
4933439C20Rik
Sc4mol
Eif2s3x
2610029G23Rik
XXF
XXYM
XYM

Fig. 3-4: Quantitative RT-PCR confirmation of feminized genes in XXY males. mRNA levels of Eif2s3x and Kdm6a (Utx) in XY males, XX females, and XXY males in both the BNST/POA and the striatum 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.

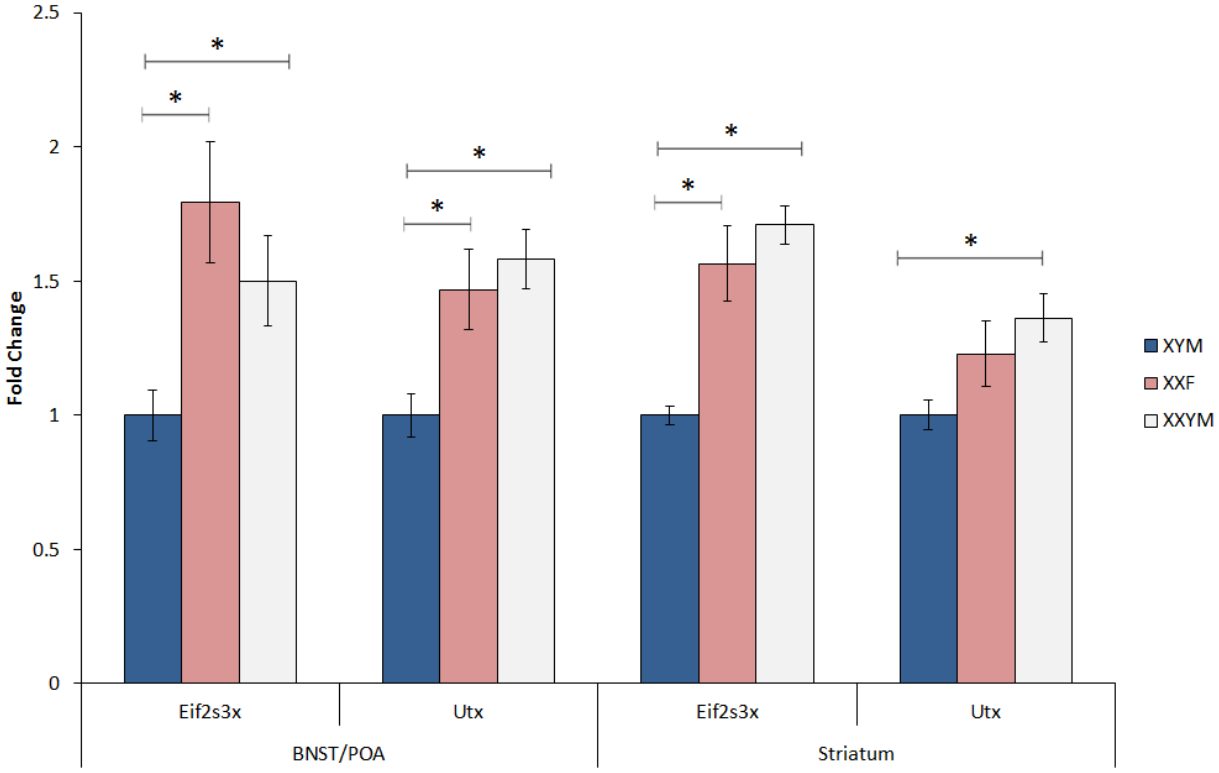


Fig 3-5: Determination of genes affected uniquely by being XXY. The Venn diagram shows genes that are differentially expressed (>1.2-fold, $p<0.05$ by Student's t-test) between XXY and XY males (yellow), and between XX and XY males (blue) in A. the BNST/POA and B. the striatum. This identified 20 genes in the BNST/POA and 19 genes in the striatum that are different between XXY and XY due to direct effects of the additional X chromosome and/or its interactions with autosomes (green).

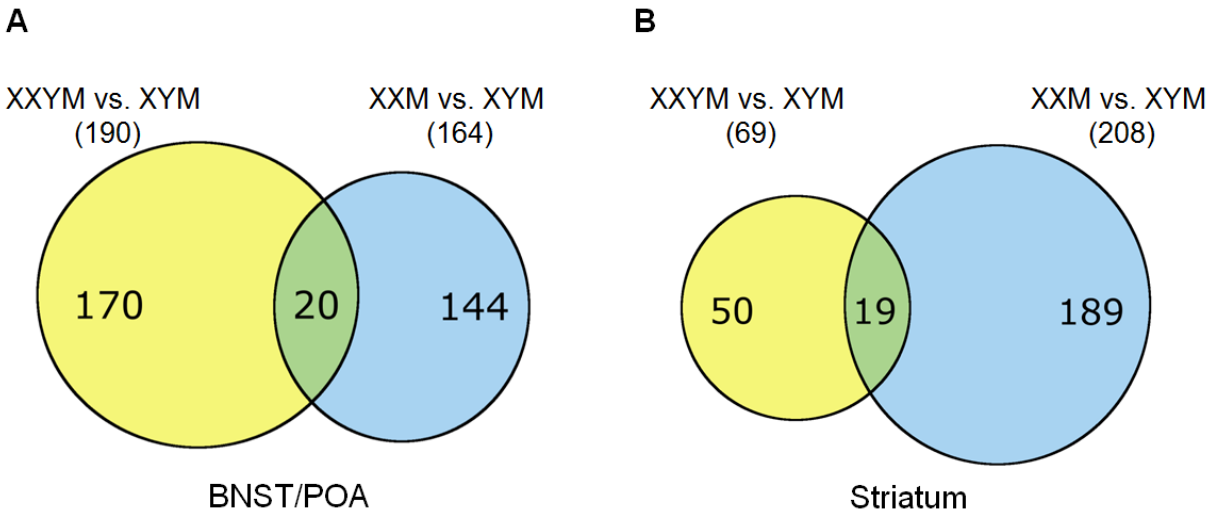


Table 3-1: Primers used in the quantitative RT-PCR validation of microarray results.

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

Table 3-2: 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 3-3: 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 3-4: 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 3-5: Top 10 pathways that are significantly affected by being XXY in the striatum ($p<0.05$ 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	Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses	3.16E-03	0.028
2	Phospholipase C Signaling	5.01E-03	0.015
3	Assembly of RNA Polymerase II Complex	9.77E-03	0.036
4	Primary Immunodeficiency Signaling	1.07E-02	0.032
5	CXCR4 Signaling	1.10E-02	0.018
6	Calcium-induced T Lymphocyte Apoptosis	1.51E-02	0.029
7	Clathrin-mediated Endocytosis Signaling	1.86E-02	0.015
8	Thrombin Signaling	2.00E-02	0.015
9	Regulation of Actin-based Motility by Rho	2.34E-02	0.023
10	Salvage Pathways of Pyrimidine Ribonucleotides	2.51E-02	0.020

Table 3-6: 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 3-5A. 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

Table 3-7: Genes that differ in both XXYM vs. XYM and XXM vs. XYM in the striatum. This corresponds to the overlap region (green) in Fig 3-5B. Most genes had the same direction of change and similar fold-change values between the two comparisons (XXYM vs. XYM, XXM vs XYM). The sole exception is *Dnalc1* which is downregulated in XXYM relative to XYM but upregulated in XXM compared to XYM.

Gene symbol	Accession	Chr	Fold change (XXYM/XYM)	Fold change (XXM/XYM)
4922503N01Rik	NM_153392.1	4	0.7	0.7
4933439C20Rik	NM_001004146.1	11	1.5	1.4
5330410G16Rik	NM_182991.2	8	0.8	0.8
Apobec1	NM_031159.3	6	0.8	0.8
Atp9b	NM_015805.2	18	0.7	0.6
BC037527	NM_172259.1	10	0.8	0.8
Bcat1	NM_007532.2	6	0.7	0.8
Dnalc1	NM_028821.1	12	0.8	1.3
Eif2s3x	NM_012010.3	X	1.4	1.6
Ercc2	NM_007949.4	0	1.3	1.3
Erdr1	NM_133362.2	0	1.8	1.8
Mcam	NM_023061.1	9	0.7	0.7
Mid1	NM_183151.1	X	3.3	1.8
Ndor1	NM_178239.2	2	0.8	0.8
Nupr1	NM_019738.1	7	1.2	1.3
Tmem25	NM_027865.1	9	0.7	0.8
Utx	NM_009483.1	X	1.3	1.6
Xist	NR_001463.2	X	140.5	127.8
Zap70	NM_009539.2	1	0.7	0.8

REFERENCES

1. Smyth, C.M. and W.J. Bremner, *Klinefelter syndrome.* Arch Intern Med, 1998. **158**(12): p. 1309-14.
2. Egan, J.F., et al., *Down syndrome births in the United States from 1989 to 2001.* Am J Obstet Gynecol, 2004. **191**(3): p. 1044-8.
3. Maclean, N., D.G. Harnden, and W.M. Court Brown, *Abnormalities of sex chromosome constitution in newborn babies.* Lancet, 1961. **2**(7199): p. 406-8.
4. Robinson, A., et al., *Sex chromosome aneuploidy: the Denver Prospective Study.* Birth defects original article series, 1990. **26**(4): p. 59-115.
5. Bojesen, A., S. Juul, and C.H. Gravholt, *Prenatal and postnatal prevalence of Klinefelter syndrome: a national registry study.* The Journal of clinical endocrinology and metabolism, 2003. **88**(2): p. 622-6.
6. Savic, I., *Advances in research on the neurological and neuropsychiatric phenotype of Klinefelter syndrome.* Current opinion in neurology, 2012. **25**(2): p. 138-43.
7. Bender, B.G., et al., *Psychosocial adaptation of 39 adolescents with sex chromosome abnormalities.* Pediatrics, 1995. **96**(2 Pt 1): p. 302-8.
8. Graham, J.M., Jr., et al., *Oral and written language abilities of XXY boys: implications for anticipatory guidance.* Pediatrics, 1988. **81**(6): p. 795-806.
9. Ratcliffe, S.G., et al., *Klinefelter's syndrome in adolescence.* Archives of disease in childhood, 1982. **57**(1): p. 6-12.
10. Ross, J.L., et al., *Behavioral and social phenotypes in boys with 47,XYY syndrome or 47,XXY Klinefelter syndrome.* Pediatrics, 2012. **129**(4): p. 769-78.

11. van Rijn, S., et al., *X Chromosomal effects on social cognitive processing and emotion regulation: A study with Klinefelter men (47,XXY).* Schizophr Res, 2006. **84**(2-3): p. 194-203.

12. van Rijn, S., et al., *What it is said versus how it is said: comprehension of affective prosody in men with Klinefelter (47,XXY) syndrome.* Journal of the International Neuropsychological Society : JINS, 2007. **13**(6): p. 1065-70.

13. van Rijn, S., et al., *Effects of an extra X chromosome on language lateralization: An fMRI study with Klinefelter men (47,XXY).* Schizophrenia Research, 2008. **101**(1-3): p. 17-25.

14. Scofield, R.H., et al., *Klinefelter's syndrome (47,XXY) in male systemic lupus erythematosus patients: support for the notion of a gene-dose effect from the X chromosome.* Arthritis and rheumatism, 2008. **58**(8): p. 2511-7.

15. Oktenli, C., et al., *Study of autoimmunity in Klinefelter's syndrome and idiopathic hypogonadotropic hypogonadism.* Journal of clinical immunology, 2002. **22**(3): p. 137-43.

16. Rovensky, J., et al., *Rheumatic diseases and Klinefelter's syndrome.* Annals of the New York Academy of Sciences, 2010. **1193**: p. 1-9.

17. Tanriverdi, F., et al., *The hypothalamic-pituitary-gonadal axis: immune function and autoimmunity.* The Journal of endocrinology, 2003. **176**(3): p. 293-304.

18. Zandman-Goddard, G., et al., *Sex and Gender Differences in Autoimmune Diseases* *Sex and Gender Aspects in Clinical Medicine,* S. Oertelt-Prigione and V. Regitz-Zagrosek, Editors. 2012, Springer London. p. 101-124.

19. Bancroft, J., D. Axworthy, and S. Ratcliffe, *The personality and psycho-sexual development of boys with 47 XXY chromosome constitution.* J Child Psychol Psychiatry, 1982. **23**(2): p. 169-80.

20. Theilgaard, A., *A psychological study of the personalities of XYY- and XXY-men.* Acta psychiatrica Scandinavica. Supplementum, 1984. **315**: p. 1-133.

21. Schiavi, R.C., et al., *Sex chromosome anomalies, hormones, and sexuality.* Arch Gen Psychiatry, 1988. **45**(1): p. 19-24.

22. Bonthuis, P.J., K.H. Cox, and E.F. Rissman, *X-chromosome dosage affects male sexual behavior.* Hormones and Behavior, 2012. **61**(4): p. 565-572.

23. Wistuba, J., et al., *Male 41, XXY* mice as a model for klinefelter syndrome: hyperactivation of leydig cells.* Endocrinology, 2010. **151**(6): p. 2898-910.

24. Liu, P.Y., et al., *Genetic, hormonal, and metabolomic influences on social behavior and sex preference of XXY mice.* Am J Physiol Endocrinol Metab, 2010. **299**(3): p. E446-55.

25. Swerdloff, R.S., et al., *Mouse model for men with klinefelter syndrome: a multifaceted fit for a complex disorder.* Acta paediatrica, 2011. **100**(6): p. 892-9.

26. Lue, Y., et al., *XXY mice exhibit gonadal and behavioral phenotypes similar to Klinefelter syndrome.* Endocrinology, 2005. **146**(9): p. 4148-54.

27. Ngun, T.C., et al., *The genetics of sex differences in brain and behavior.* Front Neuroendocrinol, 2011. **32**(2): p. 227-46.

28. Hamer, D.H., *Genetics and Male Sexual Orientation.* Science, 1999. **285**(5429): p. 803.

29. Bocklandt, S., et al., *Extreme skewing of X chromosome inactivation in mothers of homosexual men.* Human Genetics, 2006. **118**(6): p. 691-4.

30. Hines, M., et al., *Sexually dimorphic regions in the medial preoptic area and the bed nucleus of the stria terminalis of the guinea pig brain: a description and an investigation of their relationship to gonadal steroids in adulthood.* J Neurosci, 1985. **5**(1): p. 40-7.

31. Hines, M., L.S. Allen, and R.A. Gorski, *Sex differences in subregions of the medial nucleus of the amygdala and the bed nucleus of the stria terminalis of the rat.* Brain Res, 1992. **579**(2): p. 321-6.

32. Allen, L.S., et al., *Two sexually dimorphic cell groups in the human brain.* J Neurosci, 1989. **9**(2): p. 497-506.

33. Forger, N.G., et al., *Deletion of Bax eliminates sex differences in the mouse forebrain.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13666-71.

34. Lovell-Badge, R. and E. Robertson, *XY female mice resulting from a heritable mutation in the primary testis-determining gene, Tdy.* Development, 1990. **109**(3): p. 635-46.

35. Mahadevaiah, S.K., et al., *Mouse homologues of the human AZF candidate gene RBM are expressed in spermatogonia and spermatids, and map to a Y chromosome deletion interval associated with a high incidence of sperm abnormalities.* Hum Mol Genet, 1998. **7**(4): p. 715-27.

36. Eicher, E.M., et al., *The mouse Y* chromosome involves a complex rearrangement, including interstitial positioning of the pseudoautosomal region.* Cytogenet Cell Genet, 1991. **57**(4): p. 221-30.

37. Burgoyne, P.S., et al., *The Y* rearrangement in mice: new insights into a perplexing PAR.* Cytogenet Cell Genet, 1998. **80**(1-4): p. 37-40.

38. Chen, X., et al., *Sex difference in neural tube defects in p53-null mice is caused by differences in the complement of X not Y genes.* Dev Neurobiol, 2008. **68**(2): p. 265-73.

39. Arnold, A.P. and X. Chen, *What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues?* Frontiers in Neuroendocrinology, 2009. **30**(1): p. 1-9.

40. Paredes, R.G., *Evaluating the neurobiology of sexual reward.* ILAR journal / National Research Council, Institute of Laboratory Animal Resources, 2009. **50**(1): p. 15-27.

41. Shi, W., A. Oshlack, and G.K. Smyth, *Optimizing the noise versus bias trade-off for Illumina whole genome expression BeadChips.* Nucleic Acids Res, 2010. **38**(22): p. e204.

42. Johnson, W.E., C. Li, and A. Rabinovic, *Adjusting batch effects in microarray expression data using empirical Bayes methods.* Biostatistics, 2007. **8**(1): p. 118-27.

43. Shi, L., et al., *The balance of reproducibility, sensitivity, and specificity of lists of differentially expressed genes in microarray studies.* BMC bioinformatics, 2008. **9 Suppl 9**: p. S10.

44. Wijchers, P.J., et al., *Sexual dimorphism in mammalian autosomal gene regulation is determined not only by Sry but by sex chromosome complement as well.* Developmental cell, 2010. **19**(3): p. 477-84.

45. Benjamini, Y., et al., *Controlling the false discovery rate in behavior genetics research.* Behavioural Brain Research, 2001. **125**(1–2): p. 279-284.

46. Warnes, G., B. Bolker, and T. Lumley, *gplots: Various R programming tools for plotting data. R package version 2.6.0*.

47. Pirooznia, M., V. Nagarajan, and Y. Deng, *GeneVenn - A web application for comparing gene lists using Venn diagrams.* Bioinformation, 2007. **1**(10): p. 420-2.

48. Bakker, J., et al., *Sexual partner preference requires a functional aromatase (cyp19) gene in male mice.* Horm Behav, 2002. **42**(2): p. 158-71.

49. Boone, K.B., et al., *Neuropsychological profiles of adults with Klinefelter syndrome.* Journal of the International Neuropsychological Society : JINS, 2001. **7**(4): p. 446-56.

50. Ross, J.L., et al., *An extra X or Y chromosome: contrasting the cognitive and motor phenotypes in childhood in boys with 47,XYY syndrome or 47,XXY Klinefelter syndrome.* Developmental disabilities research reviews, 2009. **15**(4): p. 309-17.

51. Ross, J.L., et al., *Cognitive and motor development during childhood in boys with Klinefelter syndrome.* American journal of medical genetics. Part A, 2008. **146A**(6): p. 708-19.

52. Yang, X., et al., *Tissue-specific expression and regulation of sexually dimorphic genes in mice.* Genome Res., 2006. **16**(8): p. 995-1004.

53. Yang, F., et al., *Global survey of escape from X inactivation by RNA-sequencing in mouse.* Genome Res, 2010. **20**(5): p. 614-22.

54. Lewejohann, L., et al., *Impaired recognition memory in male mice with a supernumerary X chromosome.* Physiology & Behavior, 2009. **96**(1): p. 23-9.

55. Kreutzer, M., et al., *Axonopathy is associated with complex axonal transport defects in a model of multiple sclerosis.* Brain Pathol, 2012. **22**(4): p. 454-71.

56. Dominguez-Salazar, E., H.L. Bateman, and E.F. Rissman, *Background matters: the effects of estrogen receptor alpha gene disruption on male sexual behavior are modified by background strain.* Hormones and Behavior, 2004. **46**(4): p. 482-90.

57. Pomerantz, S.M., A.A. Nunez, and N.J. Bean, *Female behavior is affected by male ultrasonic vocalizations in house mice.* Physiol Behav, 1983. **31**(1): p. 91-6.

58. Gotsiridze, T., et al., *Development of sex differences in the principal nucleus of the bed nucleus of the stria terminalis of mice: Role of Bax-dependent cell death.* Developmental Neurobiology, 2007. **67**(3): p. 355-362.

59. Tsukahara, S., *Sex differences and roles of sex steroids in apoptosis of sexually dimorphic nuclei of preoptic area in postnatal rats.* Journal of Neuroendocrinology, 2009. **9999**(999A).

60. Fernandez-Ruiz, J.J., et al., *Nigrostriatal and mesolimbic dopaminergic activities were modified throughout the ovarian cycle of female rats.* Journal of neural transmission. General section, 1991. **85**(3): p. 223-9.

61. Xiao, L. and J.B. Becker, *Quantitative microdialysis determination of extracellular striatal dopamine concentration in male and female rats: effects of estrous cycle and gonadectomy.* Neuroscience letters, 1994. **180**(2): p. 155-8.

62. Crowley, W.R., T.L. O'Donohue, and D.M. Jacobowitz, *Changes in catecholamine content in discrete brain nuclei during the estrous cycle of the rat.* Brain research, 1978. **147**(2): p. 315-26.

63. Jori, A. and G. Cecchetti, *Homovanillic acid levels in rat striatum during the oestrous cycle.* The Journal of endocrinology, 1973. **58**(2): p. 341-2.

64. Davis, C.F., B.F. Davis, and A.E. Halaris, *Variations in the uptake of 3H-dopamine during the estrous cycle.* Life sciences, 1977. **20**(8): p. 1319-32.

65. Morissette, M. and T. Di Paolo, *Sex and estrous cycle variations of rat striatal dopamine uptake sites.* Neuroendocrinology, 1993. **58**(1): p. 16-22.

66. Di Paolo, T., P. Falardeau, and M. Morissette, *Striatal D-2 dopamine agonist binding sites fluctuate during the rat estrous cycle.* Life sciences, 1988. **43**(8): p. 665-72.

67. Becker, J.B., *Gender differences in dopaminergic function in striatum and nucleus accumbens.* Pharmacology, biochemistry, and behavior, 1999. **64**(4): p. 803-12.

68. Pohjalainen, T., et al., *Sex differences in the striatal dopamine D2 receptor binding characteristics in vivo.* The American journal of psychiatry, 1998. **155**(6): p. 768-73.

69. Dewing, P., et al., *Direct regulation of adult brain function by the male-specific factor SRY.* Current biology : CB, 2006. **16**(4): p. 415-20.

70. Haaxma, C.A., et al., *Gender differences in Parkinson's disease.* J Neurol Neurosurg Psychiatry, 2007. **78**(8): p. 819-24.

71. Harlow, T.L. and P. Gonzalez-Alegre, *High prevalence of reported tremor in Klinefelter syndrome.* Parkinsonism Relat Disord, 2009. **15**(5): p. 393-5.

72. Lenz, K.M., B.M. Nugent, and M.M. McCarthy, *Sexual differentiation of the rodent brain: dogma and beyond.* Frontiers in neuroscience, 2012. **6**: p. 26.

73. Amateau, S.K. and M.M. McCarthy, *A novel mechanism of dendritic spine plasticity involving estradiol induction of prostaglandin-E2.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2002. **22**(19): p. 8586-96.

74. Todd, B.J., J.M. Schwarz, and M.M. McCarthy, *Prostaglandin-E2: a point of divergence in estradiol-mediated sexual differentiation.* Hormones and Behavior, 2005. **48**(5): p. 512-21.

75. Crespi, B.J. and D.L. Thiselton, *Comparative immunogenetics of autism and schizophrenia.* Genes, brain, and behavior, 2011. **10**(7): p. 689-701.

76. Dantzer, R., et al., *From inflammation to sickness and depression: when the immune system subjugates the brain.* Nat Rev Neurosci, 2008. **9**(1): p. 46-56.

77. Kelley, K.W. and R. Dantzer, *Alcoholism and inflammation: Neuroimmunology of behavioral and mood disorders.* Brain, Behavior, and Immunity, 2011. **25, Supplement 1**(0): p. S13-S20.

78. Friedman, H. and T.K. Eisenstein, *Neurological basis of drug dependence and its effects on the immune system.* Journal of Neuroimmunology, 2004. **147**(1–2): p. 106-108.

79. Ando, K., et al., *N-cadherin Regulates p38 MAPK Signaling via Association with JNK-associated Leucine Zipper Protein: IMPLICATIONS FOR NEURODEGENERATION IN ALZHEIMER DISEASE.* Journal of Biological Chemistry, 2011. **286**(9): p. 7619-7628.

80. Xu, H., et al., *Regulation of Neurite Outgrowth by Interactions between the Scaffolding Protein, JNK-associated Leucine Zipper Protein, and Neuronal Growth-associated Protein Superior Cervical Ganglia Clone 10.* Journal of Biological Chemistry, 2010. **285**(6): p. 3548-3553.

81. Shi, J., et al., *The role of Brn-4 in the regulation of neural stem cell differentiation into neurons.* Neuroscience Research, 2010. **67**(1): p. 8-17.

82. Tan, X.F., et al., *The co-transduction of Nurr1 and Brn4 genes induces the differentiation of neural stem cells into dopaminergic neurons.* Cell biology international, 2011. **35**(12): p. 1217-1223.

83. Mathian, A., et al., *IFN-α Induces Early Lethal Lupus in Preautoimmune (New Zealand Black × New Zealand White)F1 but Not in BALB/c Mice.* The Journal of Immunology, 2005. **174**(5): p. 2499-2506.

84. Schild-Hay, L.J., et al., *Tamoxifen Induces Expression of Immune Response–Related Genes in Cultured Normal Human Mammary Epithelial Cells.* Cancer Research, 2009. **69**(3): p. 1150-1155.

85. Oka, T., *Prostaglandin E2 as a mediator of fever: the role of prostaglandin E (EP) receptors.* Front Biosci, 2004. **9**: p. 3046-57.

86. McCarthy, M.M. and A.P. Arnold, *Reframing sexual differentiation of the brain.* Nat Neurosci, 2011. **14**(6): p. 677-83.

87. Skuse, D.H., et al., *Evidence from Turner's syndrome of an imprinted X-linked locus affecting cognitive function.* Nature, 1997. **387**(6634): p. 705-8.

88. Loesch, D.Z., et al., *Effect of Turner's syndrome and X-linked imprinting on cognitive status: analysis based on pedigree data.* Brain Dev, 2005. **27**(7): p. 494-503.

89. Sagi, L., et al., *Clinical significance of the parental origin of the X chromosome in turner syndrome.* J Clin Endocrinol Metab, 2007. **92**(3): p. 846-52.

90. Kochi, C., et al., *The influence of parental origin of X chromosome genes on the stature of patients with 45 X Turner syndrome.* Genet Mol Res, 2007. **6**(1): p. 1-7.

91. Bruining, H., et al., *The parent-of-origin of the extra X chromosome may differentially affect psychopathology in Klinefelter syndrome.* Biol Psychiatry, 2010. **68**(12): p. 1156-62.

92. Iitsuka, Y., et al., *Evidence of skewed X-chromosome inactivation in 47,XXY and 48,XXYY Klinefelter patients.* Am J Med Genet, 2001. **98**(1): p. 25-31.

Chapter 4

The epigenetic underpinnings of brain sexual differentiation

In this project, I took the lead on the analysis of the first genome-wide scan of DNA methylation in the striatum. In terms of the data/figures, I am responsible for the design and preparation of Figure 4-1 striatum data, Figure 4-2A, Figure 4-3 data pertaining to striatum, Figure 4-4, Figure 4-5 A and B, Figure 4-7 striatum data, Table 4-1 striatum data, Table 4-2, Table 4-3A, Supplementary Figure 4-1A, Supplementary Table 4-1 striatum tab, Supplementary Table 4-2 striatum tabs, Supplementary Table 4-3 striatum tabs, Supplementary Table 4-4, Supplementary Table 4-6 striatum tab, Supplementary Table 4-7, and Supplementary Table 4-9.

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 two sexually dimorphic brain regions: the striatum, and a region that encompasses both the bed nucleus of the stria terminalis and preoptic area (BNST/POA). This was done at two different time points: postnatal day (PN) 4, which is during the sensitive period and PN60 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. 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 and not at PN4, and is more pronounced in the striatum than BNST/POA. 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 neonatal mouse striatum and BNST/POA is altered in response to steroid hormones such as testosterone and suggests 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 striatum and BNST/POA in both the 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 consisting 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 4-1) 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 striatum and BNST/POA were highly similar across all chromosomes (*Pearson coefficient*, 0.99) (Supp. Fig 4-1A and 4-1B). 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 4-1A). In addition, developmental stage-dependent loci (Fig 4-1C-E) or tissue-specific (Fig 4-1D) 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 in both the striatum (1579) and the BNST/POA (1029). Of the 1579 genes in the striatum that showed sex-specific methylation, 420 were on the X chromosome and 348 (82.86%) were more methylated in females (Fig 4-2A). Similarly, in the BNST/POA, 426 of the 1029 sex-affected genes were X-linked and 359 (84.27%) were more methylated in females (Fig 4-2B).

Focusing on the effect of sex on autosomes, we identified 1157 and 600 genes showing sex differences in the adult striatum and BNST/POA, respectively. Interestingly, a substantial

number of these genes showed higher methylation in males than females (in the striatum, male mice exhibited higher levels of methylation in 1070/1157 autosomal genes (Fig 4-2A). On the other hand, in the BNST/POA, 520/600 genes were more methylated in males relative to females during adulthood (Fig 4-2B). 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 (68 genes in the striatum and 45 genes in the BNST/POA). By day 60 of life, a much larger number of genes demonstrated methylation changes in response to testosterone (1378 and 740 genes in striatum and BNST/POA, respectively) (Fig 4-3A and Supp. Table 4-2). In both regions of the brain, a similar proportion of testosterone-affected genes exhibited sexually dimorphic methylation patterns (471/1378 or 34% in the striatum; 265/740 or 36% in the BNST/POA). 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. For example, in striatum on postnatal day 60, there was a change in methylation of 1378 genes in response to testosterone. For 907 (65%) of these genes, there were no sex-specific differences in methylation between males and females. A similar proportion of testosterone-affected genes in the PN60 BNST/POA showed no sex-specific methylation differences (475/740; 64%) (Supp. Table 4-3). These data could be attributed to a pharmacological effect of T, not normally seen in XY males or might 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 other masculinization processes that are occurring in the XY male [25].

Further assessment of 5-mC levels at testosterone-affected genes demonstrated that a substantial fraction display increased 5-mC in response to testosterone. In the striatum, 51/68 (75%) genes at PN4, and 1324/1378 (96.18%) genes at PN60 showed greater methylation in XX+T relative to XX mice. Similarly, in the BNST 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 4-3B). However, the methylation levels at several chromatin-modifying genes were found to be reduced by testosterone in the striatum. There were significant decreases in CpG methylation of *PHF20 (*a gene with histone acetyltransferase activity that acetylates histone H4 and p53), or of HENMT1 (a gene with known methyl- or O-methyltransferase activity) following testosterone administration at PN4. Testosterone effects on chromatin modifying genes were also 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.

Interestingly, among the genes whose methylation was affected by testosterone at PN4 striatum, we identified the *Fmr-1*, the fragile X mental retardation gene. Hypermethylation of 5'-upstream sequences of this gene results in the loss of fragile X mental retardation protein leading to impairments in synaptogenesis, synaptic plasticity, learning, memory, and cognition [29-31]. Consistent with our data, it has been reported that 17-β estradiol, a metabolite of testosterone, affect the methylation of *Fmr-1* upstream sequences in an age-dependent manner [32].

In the BNST/POA, 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 [33]. 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 [34]. Additionally, Bcor interacts with class I and II histone deacetylases suggesting that it achieves transcriptional silencing using these components of epigenetic machinery [35]. 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 [36, 37].

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 both regions of the brain. Our analysis revealed that testosterone alters the

methylation of genes belonging to a wide range of biological processes and functions (Supp. Table 4-4 and Supp. Table 4-5). Table 4-1 lists some of the functional categories that were enriched. Functional categories related to nervous system development were strongly represented in the datasets from both the striatum and the BNST/POA (p-value range: 1.02E-08 to 1.93E-02). Many of these functional categories were common to both regions of the brain and 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 in both regions.

However, there were also biologically relevant differences observed between the striatum and the BNST/POA. Functional categories uniquely enriched in the adult testosterone-affected striatal dataset included neurotransmission, NMDA-mediated synaptic current, action potential of cells, long term depression and development of muscle. Furthermore, an enrichment of functional categories related to neurological disease (e.g., dyskinesia, Huntington's disease, hyperactive behavior, seizures, mood disorder, ataxia, and amyotrophic lateral sclerosis) was apparent in the striatum only (Table 4-2).

Functional categories unique to the BNST/POA included quantity of neurons, cell viability of neurons, and proliferation of neuronal cells (Table 4-1). 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 [38]. The establishment of the sexually dimorphic nuclei in the BNST and the POA is reliant on highly similar cell death mechanisms [39, 40]. 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.

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 a limited number of genes whose magnitude and/or level of DNA methylation changes between XX and XX +T were similar in PN4 and PN60 (only 19 genes in the striatum and 11 genes in the BNST/POA) and classified them as stable (Table 4-3).

Notably, several distinct effects of neonatal testosterone were apparent on gene methylation levels. For a limited number of genes, the direction and the magnitude of the neonatal testosterone effect on methylation was still present at the same genomic location at PN60 (8 genes in the striatum and 4 genes in the BNST/POA), while for another subset the neonatal testosterone effects reemerged at a different location of the gene during adulthood (10 genes in the striatum and 7 genes in the BNST/POA). Unexpectedly, for some 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 (3 and 2 genes in the striatum and BNST/POA, respectively). Altogether, these data suggest that 5-mC regulation might be more dynamic than we predicted (Table 4-3).

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 in each region (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 4-4.

When we plotted histograms of where these sites in XX+T fall at each age and for each region, almost all the sexually dimorphic CpG sites were more female-like in the XX+T group at PN4 in both the BNST/POA and striatum (Fig 4-5A and Fig 4-5B). By day 60 of life, a number of sexually dimorphic CpG sites in the striatum and BNST demonstrated methylation levels more similar to males than to females although the majority remained female-like (Fig 4-5C and Fig 4-5D). Interestingly, the shift toward male values was more pronounced in the striatum compared with the BNST/POA and resembled a bimodal distribution (Fig 4-5C vs. Fig 4-5D). These data have two important implications. The first is 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. The second is that the methylome of the striatum may be more responsive to the effects of testosterone than 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) (Figure 4-6). This analysis revealed that methylation status of 4707 genes in the striatum and 3316 genes in the BNST/POA were altered between PN4 and adulthood irrespective of genotype or treatment (Figure 4-7, Supp. Table 4-6). 2553 genes were found in common between both regions.

We then analyzed the enrichment of functional categories in these age-affected genes using IPA. As expected, the majority of functional categories enriched in the two regions were shared. In the context of nervous system function, the most significantly enriched functional categories in both regions were related to the morphology of the nervous system, neuritogenesis (a detailed breakdown is shown in Supp. Table 4-7 and Supp. Table 4-8).

In addition, we identified a number of age-related statistically significant signaling pathways unique to each sex in the striatum (e.g., insulin receptor signaling, neurotrophin/TRK signaling, ERK5 signaling, IGF-1 signaling, cholecystokinin/gastrin-mediated signaling, docosahexaenoic acid (DHA) signaling, semaphorin signaling in neurons, dopamine receptor signaling, actin nucleation by ARP-WASP complex, PI3K/AKT signaling, apoptosis signaling, and DNA methylation and transcriptional repression signaling unique to females; and RhoA signaling, NF-κB signaling, phospholipid degradation, glycerophospholipid metabolism, agrin interactions at neuromuscular junction, VDR/RXR activation, and sonic hedgehog signaling

specific to males). However, the strongest age effects detected in the male and female mice were mostly similar (Supp. Table 4-9).

The patterns in the BNST/POA data were similar to those in the striatum. 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 4-10).

Discussion

Our study shows how testosterone can modify the epigenetic 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 two sexually dimorphic brain regions (the striatum and 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. We are currently annotating our data set to determine which genomic features on the X chromosome are related to the observed X chromosome hypermethylation in females.,

Testosterone altered the methylation status of a large number of CpGs particularly in the adult brain. 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 appeared much later in life and seemed to be very dynamic. These findings implied that the emergence of certain sex differences in the brain may be a gradual process that is cemented over the organism's life. However, it is important to also note

that one mechanism that might explain these findings is that the cell populations at the two ages maybe significantly different resulting in the methylation differences observed between PN4 and PN60. Second unexpected finding was that the shift towards a male-like pattern of DNA methylation in XX+T was more pronounced in the striatum than the BNST/POA. This observation was surprising since the BNST/POA displays some of the most dramatic anatomical and neurochemical sex differences in the brain that result from organization by gonadal hormones. Therefore, this raises the possibility that the dramatic masculinization of BNST/POA does not involve widespread masculinization of methylation patterns. [41]. Sex differences in the striatum are comparatively modest when using other metrics of sex differences. . Therefore, neuroanatomical and neurochemical markers of sex differences may not fully reflect the sensitivity of a brain region to the actions of gonadal hormones.

Notably, we also identified sets of testosterone-regulated loci that clearly maintained 5-mC from PN4 to 60 in each region tested although these were a small minority. 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 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. Interestingly, among the neonatal and adult striatal genes that were differentially methylated due to testosterone, a substantial number encoded signaling components associated with increased or decreased risk of Parkinson's disease (e.g. dopamine metabolism; glutamate signaling; mitochondrial function, and oxidative damage; regulation of microtubule dynamics; ubiquitin proteasome system; and autophagy-lysosome pathway). These included genes such as *Maoa*, *Park2*, *Pacrg*, *Herc3*, *Lrrk2*, *Cox19*, *Mtap7*, *Gsr*, *Gadl1*, and *Aldh18a1* that were either hyper- or hypo-methylated in XX + T vs. XX mice. For example, *Park2* (*parkin*), an E3 ubiquitin protein ligase with a protective role against α-synuclein-mediated neurotoxicity was found to be differentially methylated at PN4. This gene is the most important causative gene of autosomal recessive early-onset Parkinson disease [42]. Recent studies suggest an association between oxidative damage, nitrative, or dopaminergic stress and impairment of the function of *parkin* in sporadic PD [43-45]. *Pacrg*, a gene that shares a bidirectional promoter with *parkin* also

displayed testosterone-dependent changes in DNA methylation at PN60. PACRG protein has been shown to be present in significant levels within the astrocytic cells of the midbrain region of PD cases potentially contributing to the PD pathology [46].

Moreover, 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 during the sensitive period. Consistent with this, we found genes involved in apoptosis in the testosterone-affected dataset at both PN4 and PN60. For instance, methylation at *Bcl2* is affected by testosterone at day 60. The Bcl-2/Bax pathway has been demonstrated to play a crucial role in testosterone-modulated apoptosis [39, 40]. There was also methylation alterations driven by testosterone at the pro-survival genes *Gdnf, Xiap, Flt1*, and *NTRK2* (receptor for Bdnf) in adulthood [47-50]. Perhaps most intriguing, however, was the 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 [38, 51, 52]. 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 [38, 53, 54]. 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, 4 (*Ankrd6, Fzd9, Irx3, Vax1*) 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 [51, 55]. *Fzd9* is part of the canonical Wnt signaling pathway and appears to play a critical role in patterning the developing telencephalon [56]. It is highly expressed in neural stem cells [57]. *Irx3* is part of the highly conserves *iroquois* family of homeoproteins which participate in a wide variety of developmental processes [58]. 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 [59, 60]. Lastly, *Vax1* is expressed in the developing forebrain and helps regulate the development of the forebrain and the visual system [61]. Taken together, these data suggest that the Wnt signaling pathway may be one of the routes by which testosterone organizes sexually differentiated cell birth.

We also identified a number of genes whose methylation was altered because of age. A fraction of genes displayed age-dependent altered methylation levels across all experimental groups, while a subset exhibited age-dependent methylation changes in a group-specific manner in both the striatum and BNST/POA.

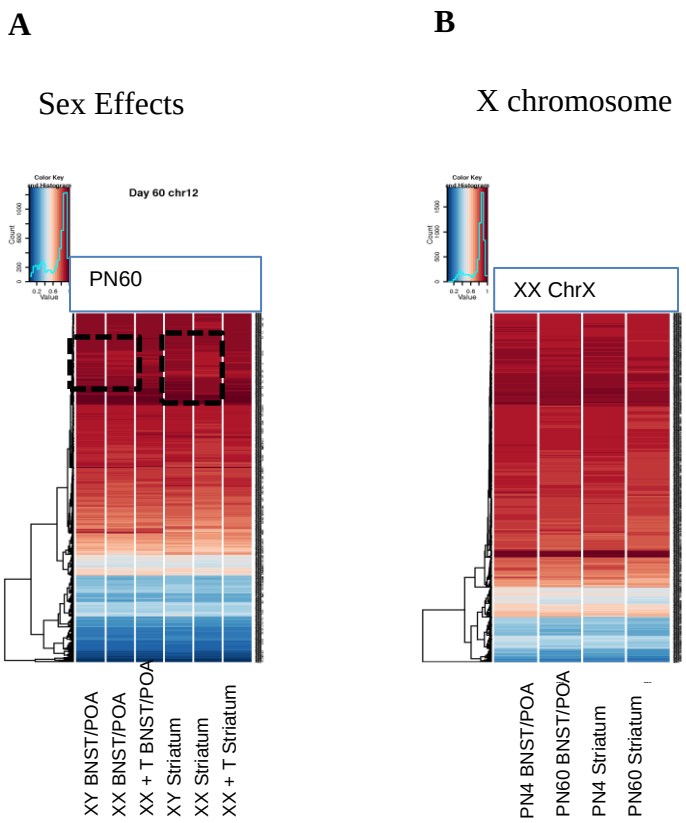
With our current study, we bring further evidence for the importance of the testosterone in regulation of methylation. Taken together, our results suggest that early testosterone exposure has broad effects on brain methylation patterns particularly during adulthood. However, it is worthwhile to note 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 4-1: 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 striatum and 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-, developmental stage-, and tissue-specific methylation differences are shown in the dashed boxes.



C

Developmental Stage Effects

D

Tissue-Specific Effects

E

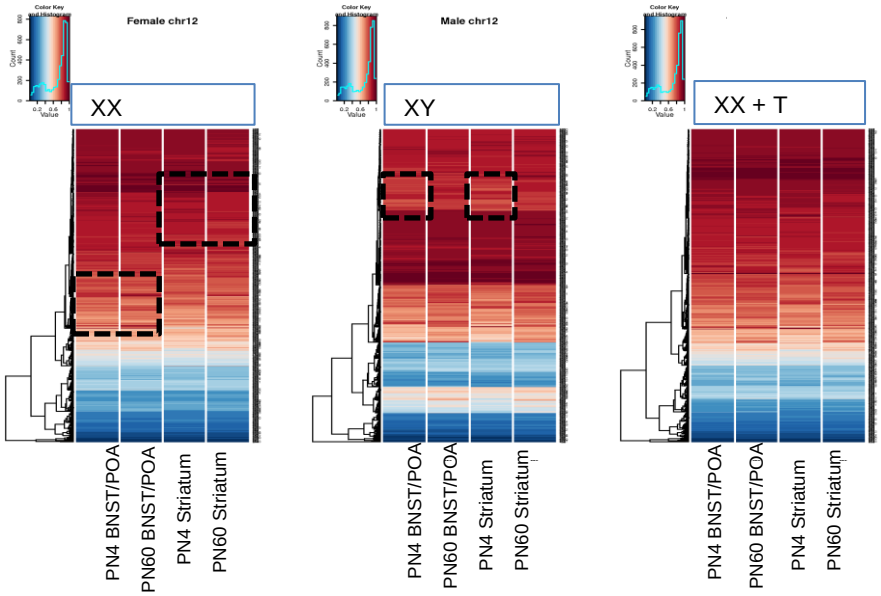


Figure 4-2: Displayed are the fractions of X, Y, and autosomal genes displaying higher methylation in one sex or the other in **A.** P60 striatum, and **B.** P60 BNST/POA. Note that in striatum only one Y chromosome gene was found to be differentially methylated while in BNST/POA, no Y encoded differentially methylated gene was identified.

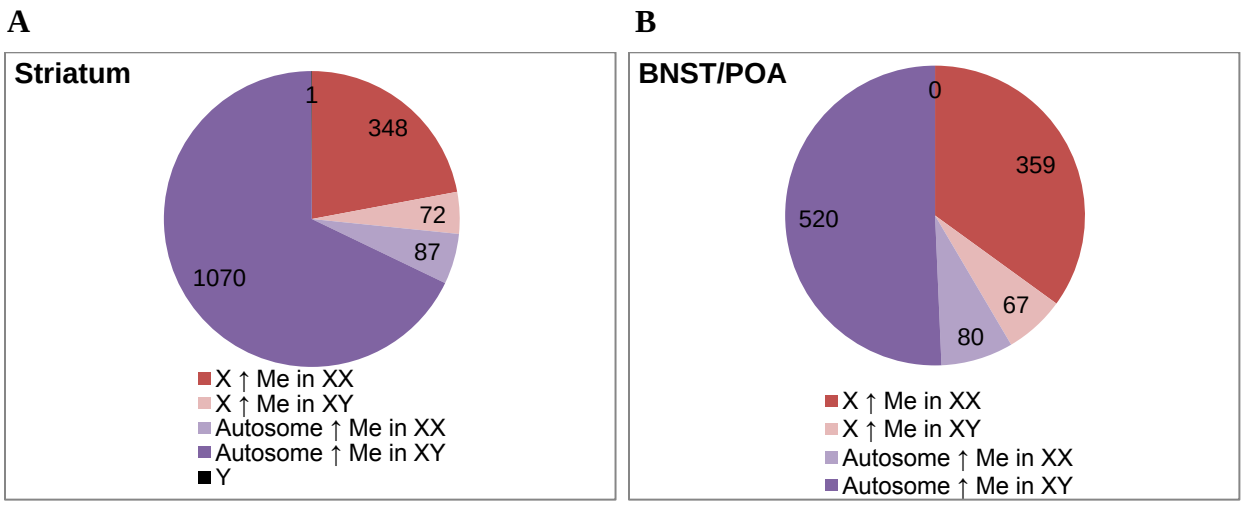
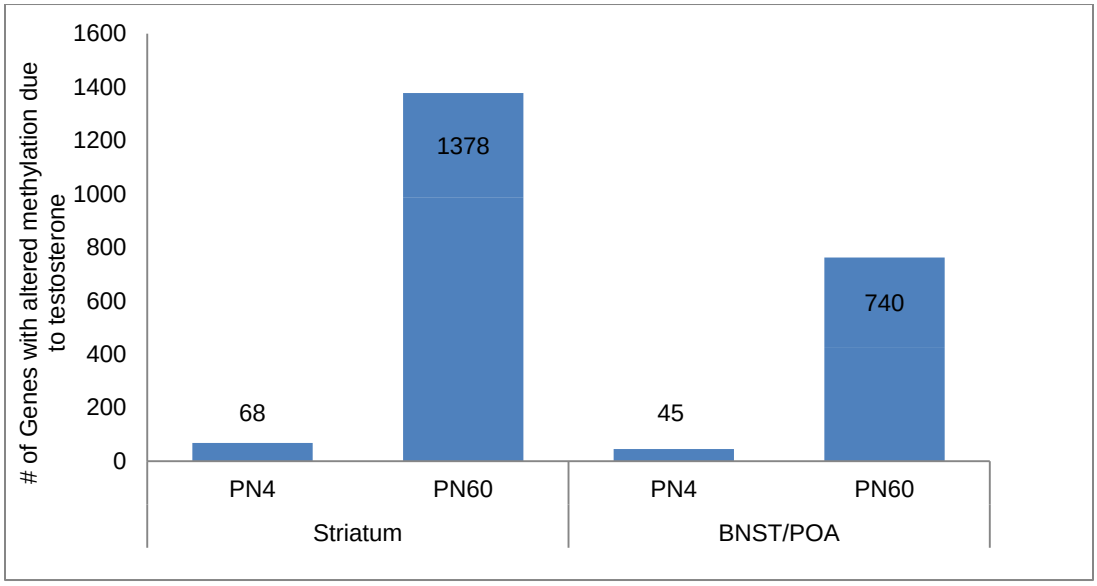


Figure 4-3: A. The number of genes affected by perinatal testosterone exposure. A. Number of genes where methylation is altered by testosterone in PN4 and PN60 striatum, and P4 and PN60 BNST/POA. **B.** Fraction of genes that exhibit testosterone-dependent hypo- or hyper-methylation at each age, in each tissue.

A



B

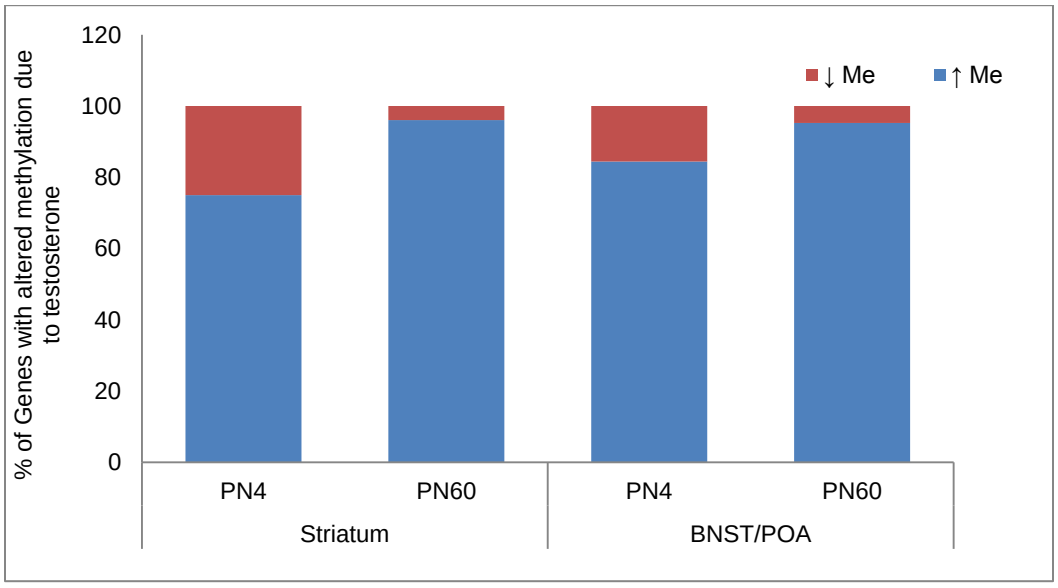


Figure 4-4: Schematic representations of several potential scenarios by which testosterone affects CpG methylation in the brain of female mice plotted on a continuum between male and female methylation (rescaled to 0 and 100, respectively).

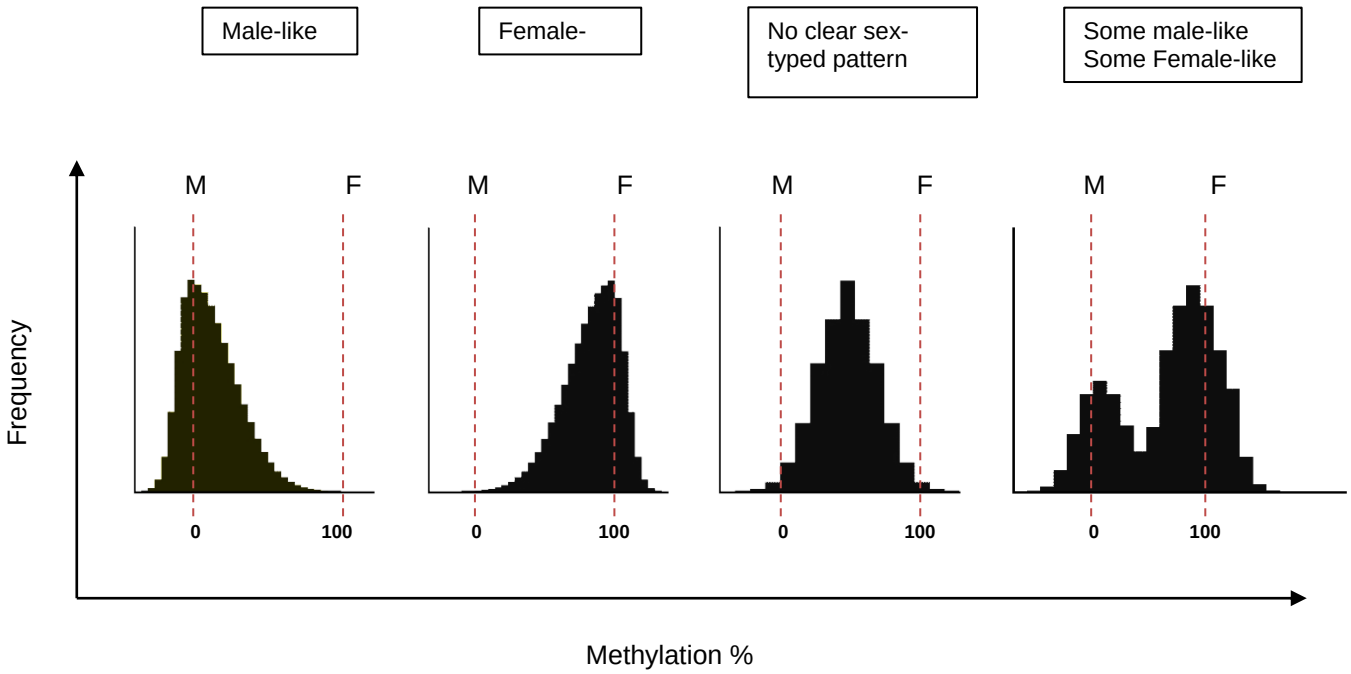


Figure 4-5: DNA methylation patterns are more masculine in XX + T at PN60. Mean XX + T methylation of the genes that display significant basal sex differences (delta methylation$_{(XX-XXY)}$ ≥15%, p value ≤ 0.05) are plotted on a continuum between XY (0) and XX (100) methylation levels in **A.** PN4 and **C.** PN60 striatum , and **B.** PN4 and **D.** PN60 BNST/POA .

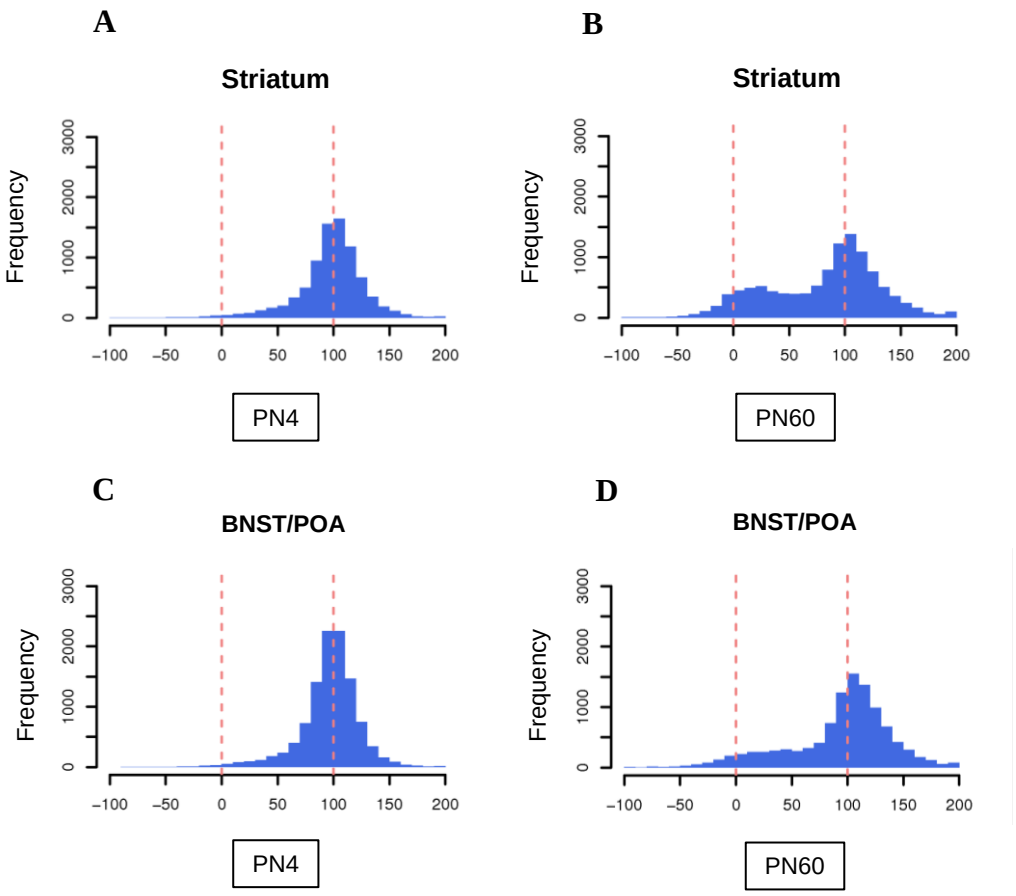


Figure 4-6: Global DNA methylation at each age is represented in this figure. All samples at each age were considered together without regard for experimental group. Statistical analysis was done by student's t test.

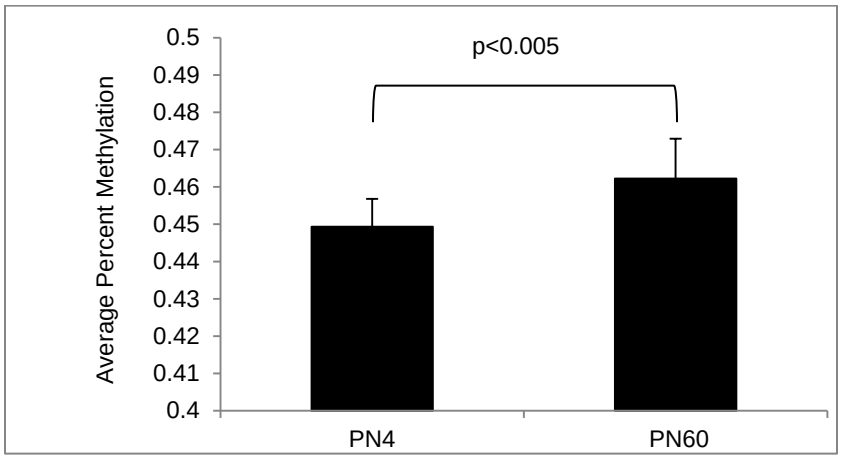


Figure 4-7: Fraction of genes that exhibit age-dependent altered methylation levels across all experimental groups, vs. those that display age-dependent methylation changes in a group-specific manner in both the striatum and BNST/POA.

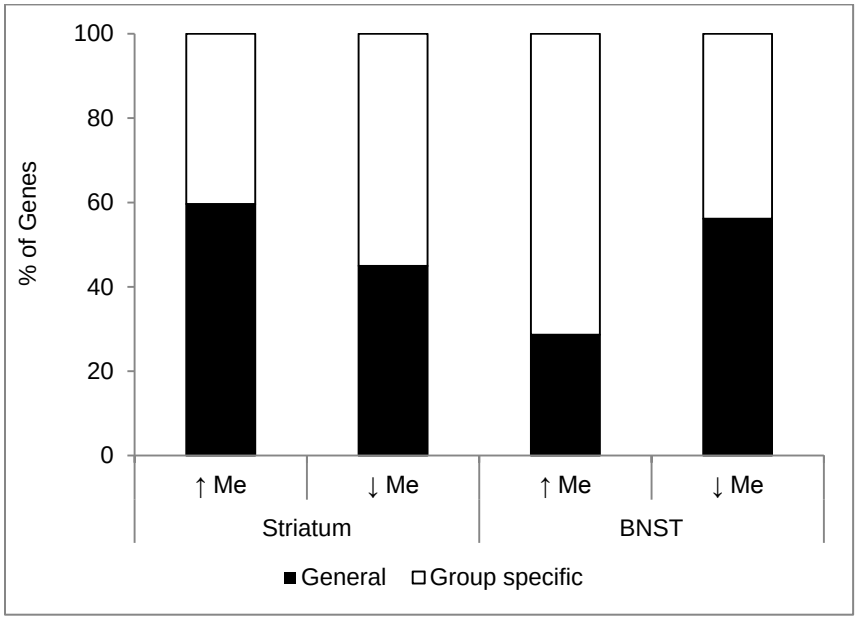


Table 4-1: Examples of some of the functional categories enriched in the testosterone data-set for both the striatum and BNST/POA.

Category	Function Annotation	Striatum		BNST/POA	
		p-value	# Genes	p-value	# Genes
Nervous System Development and Function	Morphology of nervous system	1.02E-08	108	3.91E-06	63
	Development of central nervous system	6.63E-08	80	4.71E-08	54
	Morphology of nervous tissue	7.88E-06	72	7.75E-05	44
	Neuritogenesis	1.05E-05	48	1.03E-07	37
	Outgrowth of neurites	4.68E-05	39	2.19E-03	22
	Coordination	4.69E-05	31	1.67E-02	15
	Axonogenesis	1.48E-04	24	1.18E-04	17
	Excitatory postsynaptic potential	1.51E-04	18	5.55E-03	10
	Growth of neurites	1.75E-04	41	5.21E-03	23
	Morphology of neurites	7.77E-04	23	1.99E-04	17
	Morphology of dendritic spines	7.78E-04	5	7.85E-04	4
	Guidance of axons	9.29E-04	21	1.60E-05	18
	Outgrowth of axons	9.56E-04	14	9.82E-03	8
	Neurotransmission	6.32E-05	44	-	-
	NMDA-mediated synaptic current	2.93E-04	6	-	-
	Action potential of cells	3.58E-04	18	-	-
	Long-term depression	1.30E-03	13	-	-
	Quantity of neurons	-	-	3.81E-05	24
	Cell viability of neurons	-	-	1.93E-02	12
Cellular Assembly and Organization	Organization of cytoskeleton	1.86E-07	125	1.72E-06	77
	Microtubule dynamics	2.17E-07	108	7.64E-06	65
Skeletal and Muscular System Development and Function	Development of muscle	2.55E-06	49	-	-
Cell Death and Survival	Apoptosis	7.68E-06	250	1.70E-03	138
Cellular Growth and Proliferation	Proliferation of neuronal cells	-	-	1.25E-02	13
Behavior	Learning	3.33E-03	38	2.81E-04	27
	Social behavior	-	-	2.59E-03	7

Table 4-2: Examples of top "neurological disease" functional categories that are significantly enriched in the list of testosterone-influenced genes in the striatum.

Function Annotation	p-value	# Genes
Movement disorder	1.03E-04	118
Congenital anomaly of brain	6.81E-04	26
Dyskinesia	3.36E-03	73
Huntington's disease	3.43E-03	70
Hyperactive behavior	3.47E-03	18
Schizophrenia	3.90E-03	56
Jervell and Lange-Nielsen syndrome	4.66E-03	2
Seizures	5.08E-03	33
Mood disorder	5.35E-03	50
Hydrocephalus	6.76E-03	11
Ataxia	6.89E-03	22
Amyotrophic lateral sclerosis	7.00E-03	18
Incoordination	9.03E-03	3
Oligodendroglioma	9.64E-03	5
Spina bifida	1.33E-02	7
Degeneration of brain	1.34E-02	8

Table 4-3: List of genes whose magnitude and/or level of DNA methylation changes between XX and XX +T were similar in PN4 and PN60. Negative delta methylation indicates higher methylation in XX + T. **A.** Striatum; **B.** BNST/POA.

A

Striatum

Gene Symbol	Δ Me at PN4	Fragment_Coordinate at PN4	Δ Me at PN60	Fragment_Coordinate at PN60
4921515J06Rik	0.22	chr3:108742959-108743236	-0.13	chr3:108742959-108743236
Taf4b	-0.11	chr18:15048075-15048318	-0.1	chr18:15048075-15048318
F8a	-0.1	chrX:70473904-70474128	-0.13	chrX:70474305-70474419
Fmr1	-0.1	chrX:65932427-65932537	-0.11	chrX:65932427-65932537
Kcnq1	0.28	chr7:150455734-150456023	-0.14	chr7:150481372-150481523
Rbbp7	-0.23	chrX:159198854-159199070	-0.12	chrX:159198688-159198839
Sox3	-0.11	chrX:58145676-58145842	-0.11	chrX:58146499-58146619
Dab1	-0.1	chr4:104298501-104298709	-0.11	chr4:104275571-104275789
Nnat	-0.13	chr2:157385832-157386011	-0.12	chr2:157386045-157386214
Arid3b	-0.19	chr9:57685767-57685957	-0.11	chr9:57685767-57685957
Grip1	-0.21	chr10:119402892-119403155	-0.12	chr10:119402892-119403155
Lonrf3	-0.1	chrX:33868422-33868652	-0.12	chrX:33869078-33869231
Clybl	-0.15	chr14:122662639-122662815	-0.12	chr14:122629995-122630146
Sorcs2	-0.13	chr5:36720053-36720329	-0.12	chr5:36511861-36512058
2610018G03Rik	-0.11	chrX:48194982-48195124	-0.12	chrX:48194982-48195124
Rap2c	-0.17	chrX:48370998-48371218	-0.12	chrX:48370998-48371218
Fndc3b	0.12	chr3:27382887-27383015	-0.21	chr3:27368716-27368995
Ubash3b	-0.12	chr9:40872064-40872268	-0.14	chr9:40872064-40872268
Foxk1	-0.25	chr5:142921051-142921281	-0.27	chr5:142921051-142921281

B

BNST/POA

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

References

1. Harrison, P. and E. Tunbridge, *Catechol-O-Methyltransferase (COMT): A Gene Contributing to Sex Differences in Brain Function, and to Sexual Dimorphism in the Predisposition to Psychiatric Disorders.* Neuropsychopharmacology, 2008. **33**: p. 3037-3045.
2. Holden, C., *Sex and the suffering brain.* Science, 2005. **308**(5728): p. 1574.
3. Murray, H., et al., *Dose- and sex-dependent effects of the neurotoxin 6-hydroxydopamine on the nigrostriatal dopaminergic pathway of adult rats: differential actions of estrogen in males and females.* Neuroscience, 2003. **116**(213-222).
4. Baron-Cohen, S., R.C. Knickmeyer, and M.K. Belmonte, *Sex differences in the brain: implications for explaining autism.* Science, 2005. **310**(5749): p. 819-23.
5. Swerdlow, R.H., et al., *Gender ratio differences between Parkinson's disease patients and their affected relatives.* Parkinsonism & related disorders, 2001. **7**(2): p. 129-133.
6. Arnold, A.P. and R.A. Gorski, *Gonadal steroid induction of structural sex differences in the central nervous system.* Annual review of neuroscience, 1984. **7**: p. 413-42.
7. Phoenix, C.H., et al., *Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig.* Endocrinology, 1959. **65**: p. 369-82.
8. McCarthy, M.M., *Estradiol and the developing brain.* Physiological reviews, 2008. **88**(1): p. 91-124.
9. Honda, S., et al., *Disruption of sexual behavior in male aromatase-deficient mice lacking exons 1 and 2 of the cyp19 gene.* Biochemical and biophysical research communications, 1998. **252**(2): p. 445-9.

10. Ogawa, S., et al., *Abolition of male sexual behaviors in mice lacking estrogen receptors alpha and beta (alpha beta ERKO).* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(26): p. 14737-41.

11. Ogawa, S., et al., *Roles of estrogen receptor-alpha gene expression in reproduction-related behaviors in female mice.* Endocrinology, 1998. **139**(12): p. 5070-81.

12. Kudwa, A.E., et al., *A previously uncharacterized role for estrogen receptor beta: defeminization of male brain and behavior.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(12): p. 4608-12.

13. Juntti, S.A., et al., *The androgen receptor governs the execution, but not programming, of male sexual and territorial behaviors.* Neuron, 2010. **66**(2): p. 260-72.

14. Dewing, P., et al., *Sexually dimorphic gene expression in mouse brain precedes gonadal differentiation.* Brain research. Molecular brain research, 2003. **118**(1-2): p. 82-90.

15. Arnold, A.P., *Experimental analysis of sexual differentiation of the zebra finch brain.* Brain research bulletin, 1997. **44**(4): p. 503-7.

16. Wade, J. and A.P. Arnold, *Functional testicular tissue does not masculinize development of the zebra finch song system.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(11): p. 5264-8.

17. Ngun, T.C., et al., *The genetics of sex differences in brain and behavior.* Frontiers in Neuroendocrinology, 2011. **32**(2): p. 227-46.

18. Reik, W., *Stability and flexibility of epigenetic gene regulation in mammalian development.* Nature, 2007. **447**(7143): p. 425-32.

19. Suzuki, M.M. and A. Bird, *DNA methylation landscapes: provocative insights from epigenomics.* Nature reviews. Genetics, 2008. **9**(6): p. 465-76.

20. Zemach, A., et al., *Genome-wide evolutionary analysis of eukaryotic DNA methylation.* Science, 2010. **328**(5980): p. 916-9.

21. Jaenisch, R. and A. Bird, *Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals.* Nature genetics, 2003. **33 Suppl**: p. 245-54.

22. Nugent, B.M., J.M. Schwarz, and M.M. McCarthy, *Hormonally mediated epigenetic changes to steroid receptors in the developing brain: implications for sexual differentiation.* Hormones and behavior, 2011. **59**(3): p. 338-44.

23. Gu, H., et al., *Genome-scale DNA methylation mapping of clinical samples at single-nucleotide resolution.* Nature methods, 2010. **7**(2): p. 133-6.

24. Schwarz, J.M., B.M. Nugent, and M.M. McCarthy, *Developmental and hormone-induced epigenetic changes to estrogen and progesterone receptor genes in brain are dynamic across the life span.* Endocrinology, 2010. **151**(10): p. 4871-81.

25. De Vries, G.J., *Minireview: Sex differences in adult and developing brains: compensation, compensation, compensation.* Endocrinology, 2004. **145**(3): p. 1063-8.

26. Sundqvist, A., K. Sollerbrant, and C. Svensson, *The carboxy-terminal region of adenovirus E1A activates transcription through targeting of a C-terminal binding protein-histone deacetylase complex.* FEBS Letters, 1998. **429**(2): p. 183-188.

27. Sural, T.H., et al., *The MSL3 chromodomain directs a key targeting step for dosage compensation of the Drosophila melanogaster X chromosome.* Nat Struct Mol Biol, 2008. **15**(12): p. 1318-25.

28. Kim, D., et al., *Corecognition of DNA and a methylated histone tail by the MSL3 chromodomain.* Nat Struct Mol Biol, 2010. **17**(8): p. 1027-9.

29. Garber, K.B., J. Visootsak, and S.T. Warren, *Fragile X syndrome.* European journal of human genetics : EJHG, 2008. **16**(6): p. 666-72.

30. Bardoni, B., et al., *The fragile X syndrome: exploring its molecular basis and seeking a treatment.* Expert reviews in molecular medicine, 2006. **8**(8): p. 1-16.

31. Zalfa, F., T. Achsel, and C. Bagni, *mRNPs, polysomes or granules: FMRP in neuronal protein synthesis.* Current opinion in neurobiology, 2006. **16**(3): p. 265-9.

32. Singh, K. and S. Prasad, *Age- and sex-related analysis of methylation of 5'-upstream sequences of Fmr-1 gene in mouse brain and modulation by sex steroid hormones.* Biogerontology, 2008. **9**(6): p. 455-65.

33. Ngun, T.C., et al., *The genetics of sex differences in brain and behavior.* Front Neuroendocrinol, 2011. **32**(2): p. 227-46.

34. Gearhart, M.D., et al., *Polycomb group and SCF ubiquitin ligases are found in a novel BCOR complex that is recruited to BCL6 targets.* Mol Cell Biol, 2006. **26**(18): p. 6880-9.

35. Huynh, K.D., et al., *BCoR, a novel corepressor involved in BCL-6 repression.* Genes & Development, 2000. **14**(14): p. 1810-1823.

36. Maine, G.N., et al., *COMMD1 promotes the ubiquitination of NF-kappaB subunits through a cullin-containing ubiquitin ligase.* EMBO J, 2007. **26**(2): p. 436-47.

37. Grimm, S., et al., *Bcl-2 down-regulates the activity of transcription factor NF-kappaB induced upon apoptosis.* The Journal of Cell Biology, 1996. **134**(1): p. 13-23.

38. Ahmed, E.I., et al., *Pubertal hormones modulate the addition of new cells to sexually dimorphic brain regions.* Nat Neurosci, 2008. **11**(9): p. 995-7.

39. Tsukahara, S., *Sex differences and roles of sex steroids in apoptosis of sexually dimorphic nuclei of preoptic area in postnatal rats.* Journal of Neuroendocrinology, 2009. **9999**(999A).

40. Gotsiridze, T., et al., *Development of sex differences in the principal nucleus of the bed nucleus of the stria terminalis of mice: Role of Bax-dependent cell death.* Developmental Neurobiology, 2007. **67**(3): p. 355-362.

41. Jacobson, C.D. and R.A. Gorski, *Neurogenesis of the sexually dimorphic nucleus of the preoptic area in the rat.* J Comp Neurol, 1981. **196**(3): p. 519-29.

42. Dawson, T.M. and V.L. Dawson, *Molecular pathways of neurodegeneration in Parkinson's disease.* Science, 2003. **302**(5646): p. 819-22.

43. LaVoie, M.J., et al., *Dopamine covalently modifies and functionally inactivates parkin.* Nat Med, 2005. **11**(11): p. 1214-21.

44. Chung, K.K., et al., *S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function.* Science, 2004. **304**(5675): p. 1328-31.

45. Yao, D., et al., *Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity.* Proc Natl Acad Sci U S A, 2004. **101**(29): p. 10810-4.

46. Taylor, J.M., et al., *Parkin Co-Regulated Gene (PACRG) is regulated by the ubiquitin-proteasomal system and is present in the pathological features of Parkinsonian diseases.* Neurobiology of disease, 2007. **27**(2): p. 238-47.

47. Decaussin, M., et al., *Expression of vascular endothelial growth factor (VEGF) and its two receptors (VEGF-R1-Flt1 and VEGF-R2-Flk1/KDR) in non-small cell lung*

carcinomas (NSCLCs): correlation with angiogenesis and survival. The Journal of Pathology, 1999. **188**(4): p. 369-377.

48. Loeliger, M.M., T. Briscoe, and S.M. Rees, *BDNF Increases Survival of Retinal Dopaminergic Neurons after Prenatal Compromise.* Investigative Ophthalmology & Visual Science, 2008. **49**(3): p. 1282-1289.

49. Lessard, C.B., S.L. Wagner, and E.H. Koo, *And four equals one: Presenilin takes the γ-secretase role by itself.* Proceedings of the National Academy of Sciences, 2010. **107**(50): p. 21236-21237.

50. Drummond, E.S., et al., *Altered Expression of Alzheimer's Disease-Related Proteins in Male Hypogonadal Mice.* Endocrinology, 2012. **153**(6): p. 2789-2799.

51. Fuso, A., et al., *S-adenosylmethionine reduces the progress of the Alzheimer-like features induced by B-vitamin deficiency in mice.* Neurobiology of Aging, 2012. **33**(7): p. 1482.e1-1482.e16.

52. Schulz, K.M., H.A. Molenda-Figueira, and C.L. Sisk, *Back to the future: The organizational-activational hypothesis adapted to puberty and adolescence.* Horm Behav, 2009. **55**(5): p. 597-604.

53. Chung, W.C., G.J. De Vries, and D.F. Swaab, *Sexual differentiation of the bed nucleus of the stria terminalis in humans may extend into adulthood.* J Neurosci, 2002. **22**(3): p. 1027-33.

54. Pinos, H., et al., *The development of sex differences in the locus coeruleus of the rat.* Brain Research Bulletin, 2001. **56**(1): p. 73-78.

55. Fuso, A., et al., *Changes in Presenilin 1 gene methylation pattern in diet-induced B vitamin deficiency.* Neurobiology of Aging, 2011. **32**(2): p. 187-199.

56. Harrison-Uy, S.J. and S.J. Pleasure, *Wnt signaling and forebrain development.* Cold Spring Harb Perspect Biol, 2012. **4**(7): p. a008094.

57. Yun, S.J., et al., *Transcriptional regulatory networks associated with self-renewal and differentiation of neural stem cells.* J Cell Physiol, 2010. **225**(2): p. 337-47.

58. Gómez-Skarmeta, J.L. and J. Modolell, *Iroquois genes: genomic organization and function in vertebrate neural development.* Current Opinion in Genetics & Development, 2002. **12**(4): p. 403-408.

59. Briscoe, J., et al., *A Homeodomain Protein Code Specifies Progenitor Cell Identity and Neuronal Fate in the Ventral Neural Tube.* Cell, 2000. **101**(4): p. 435-445.

60. Janssens, S., et al., *Direct control of Hoxd1 and Irx3 expression by Wnt/beta-catenin signaling during anteroposterior patterning of the neural axis in Xenopus.* Int J Dev Biol, 2010. **54**(10): p. 1435-42.

61. Hallonet, M., et al., *Vax1, a novel homeobox-containing gene, directs development of the basal forebrain and visual system.* Genes & Development, 1999. **13**(23): p. 3106-3114.

Chapter 5

Conclusion

WHY STUDY SEX DIFFERENCES?

Whether one is a man or a woman has a profound influence on their physiology and how they experience different diseases – incidence rate, prevalence, age of onset, and severity of symptoms can all differ depending on one's biological sex. Such sex differences have been widely noted in various autoimmune, cardiovascular, and cancerous diseases. In addition, many sex differences have been found in mental disorders with women having a higher prevalence of depression and anxiety disorders, and men having higher rates of diseases such as schizophrenia, ADHD, and autism [1, 2].

However, well over 50% of scientific studies published so far do not consider the impact of sex. Generally, these studies are conducted only on one sex, often the wrong one or in some instances the sex is not even specified [3]. Even if the sex is indicated, the study focuses on very highly sexually dimorphic brain regions that are particularly involved in control of sex behavior leading to the false notion that relatively few sex specific brain phenotypes exist.

Thus, keeping in mind how most diseases affect men and women differently and how men and women exhibit anatomical, physiological and behavioral sex differences, it becomes crucial to better understand the underlying physiology and reasons behind these sex differences, and to properly include women in research studies so that it reflects their *true* numbers in the population.

The requirement for reporting of sex-specific scientific results is being recognized more and more every day and in fact, more credit is given to manuscripts or studies that contain sex-specific information, and conduct sex-stratified analyses. Since 1998, the investigational new drug (IND) application and new drug application (NDA) regulation requires applicants to

provide numbers of subjects in relation to their sex. The numbers of clinical trials that report results according to sex are increasing. More and more drug labels contain sex-specific pharmacokinetic information.

Altogether, identifying the factors that contribute to sex differences, and determining the magnitude of their effect is of utmost importance. In this respect, journals and funding agencies should persuade authors to include sex-related information in their manuscripts; NIH should give higher priority to those proposals that include both sexes; and the FDA should generate safeguards to make certain that clinicians and the public are informed of the sex differences in drug reactions and dosages [4]. In addition, core training in the neuroscience graduate programs and medical school should also include a course that will highlight the significance of brain sex differences. New experimental paradigms (e.g. high-throughput epigenomic and molecular genetic approaches; targeted imaging, etc.) should model sex-specific hormonal, genetic, epigenetic, developmental stage, and environmental influences on gene expression and signaling pathways in the brain. Novel methodologies should be devised to define similarities and disparities in sexually differentiated brain function.

OVERARCHING GOALS OF THIS RESEARCH

In this proposal, we sought to identify the main determinants of sexual dimorphism in the brain. Since the exact molecular mechanisms by which hormones and/or sex chromosome genes contribute to sex differences in the brain remains a poorly understood and relatively unexplored research area, we first asked whether the organizational effects of hormones on the developing brain could be mediated via epigenetic mechanisms. Next, we examined the role that sex chromosomal genes play in producing brain sex differences, particularly brain gene expression

and determined how differences in the number of sex chromosomes could affect sexually dimorphic behavior.

ORGANIZATIONAL EFFECTS OF HORMONES

The groundbreaking work of Phoenix et al. (1959) has changed the way we view sex differences in brain and behavior. Their study showed that sex differences in behavior are permanently organized by testosterone, during an early critical period of development and like the genital tracts, the early testosterone effects are detected long after the end of initial exposure. Upon hormonal surges during puberty, steroid hormones affect the previously sexually organized circuits to enhance the expression of sex-specific behaviors. According to this theory, both sexes have the potential to show behaviors typical of the opposite sex, however upon exposure to the hormones produced during the critical period, sex-specific capacities are readily and irreversibly organized [5].

The idea that the differences in the adult behaviors are the result of differences in capacity initiated by the action of testosterone during critical periods has been continuously examined and remarkably reconfirmed in many cellular, molecular, and genetic studies of sex differences. This approach has been repeatedly used in studying the sex differences in the volumes of brain regions that are important for the control of reproductive behavior, or the number or size of cells in those regions and has repeatedly shown that hormones are the major determinant of these sex differences. In addition, smaller sex differences in brain regions, which are involved in behaviors not as sexually dimorphic as the reproductive behavior (e.g., thickness of the cerebral cortex, sex-typical responses to stress and nociceptive stimuli, and sex differences in learning and cognition) have also been examined and attributed to gonadal hormone effects [6-12].

Remarkably, the general framework of the organizational/activational theory of brain sexual differentiation has withstood the test of time, with some new additions to the theory. For example, it is now known that organizational hormone effects are not limited to just the highly sexually differentiated regions of the brain; regions and cells that receive projections from these highly sexually differentiated regions also process the new information with regards to sex in the brain, arguing against the idea that the brain is largely sexually monomorphic.

There are also differing views of how the organizational hormones act on the brain. One view is that the hormonally-mediated brain sexual differentiation is a slow process due to the fact that hormones have to travel to distant areas of the body, affect the cellular processes there, and make small changes in the rate of synthesis and degradation of signaling molecules causing permanent sexual differentiation [13, 14]. Although that view still has credence, it is also true that hormones can be synthesized locally in the brain or can act rapidly on membrane-bound receptors and generate signal transduction pathways that cause active changes in the cell's physiology [15]. Detailed characterization of steroid hormone effects and mechanisms involved has provided evidence for the involvement of both slow and rapid onset of hormonal action. Furthermore, recent studies of mice with null mutations for steroid receptors confirm the effects of hormones, but also yield multiple molecular pathways that respond to estrogens and androgens. Not only does each cellular pathway occur via different molecular pathways, but the various brain regions have different sex-specific cellular responses to the same gonadal hormones (e.g., modulation of cell proliferation, cell survival, cell phenotype, connectivity) [16-21]. Altogether, these data elute to the complex relationship between hormones and brain sexual differentiation. Further characterization of the factors mediating the effects of perinatal testosterone on brain organization would fill the gap in our perception of how interactions

between gonadal hormones and the neonatal brain determine sex differences in adolescence and adulthood.

Our work with regards to organizational effects and epigenetics

Development during the critical periods requires the stable modulation of gene expression, which all the while is being affected by epigenetic processes such as histone modifications and DNA methylation. Chromatin structure and DNA-modifying enzymes are regulated and in turn regulate the nervous system development and function. In fact, many recent studies have found that early environmental conditions can alter DNA methylation and histone modification patterns, leading to lifelong changes in the brain, which in some instances can be transgenerational [22]. Therefore, we sought to determine whether early testosterone exposure could exert long-term effects on methylation, in such a way that the influence of this experience could be observed later on in adulthood.

We employed a single nucleotide-resolution method that produces results for CpG sites within CpG islands, promoters and enhancer elements with high coverage. Using a +/- 10% differential methylation cutoff criterion, a substantial number of genes in the striatum of female mice underwent dynamic methylation changes after a single injection of testosterone on the day of birth. More than one-third of these genes also displayed sex differences in their methylation patterns. On the other hand, a subset of testosterone-influenced genes, did not exhibit sexually dimorphic methylation, either because of the pharmacological effects of testosterone, the interplay between the sex chromosome complement and testosterone, or because of testosterone's potential to counteract the effects of other sex-specific signals to keep the two sexes as equivalent as possible.

Using Ingenuity Pathway Analysis, we determined the biological processes and functional pathways that were influenced by testosterone-regulated methylation and found that many of the testosterone-affected genes were mainly related to brain and nervous system development. Neurological disease categories related to movement disorders, particularly those with a higher prevalence in males, were also enriched in the adult testosterone striatal data set. This was consistent with striatum's role in regulation of movement and established a link between testosterone and regulation of movement genes through DNA methylation.

Our work also provided the evidence that the sexually dimorphic CG sites were more female-like during the neonatal period whereas by adulthood, there was a shift to a more male-like pattern. These data were consistent with the previously substantial methylation changes we observed during adulthood and hinted at the idea that testosterone-driven masculinizing effects on methylation are not immediately evident but arise later in life. According to the previous studies of brain organization, phenotypic sex differences are established during the perinatal period and persist into adulthood. Our data provided a new perspective regarding the role that testosterone plays in brain sexual differentiation. It implied that the neural molecular patterns found in adults are not the result of immediate actions of testosterone on the brain. Rather, these molecular marks are conditioned by early hormonal exposure, the effects of which emerge over a period of time.

Since brain sexual differentiation is also regulated by direct genetic effects, we found those genes that were differentially methylated between males and females but not affected by testosterone. As with testosterone-dependent methylation, many more genes were testosterone-independent in adult compared to neonatal animals. When looking at the biological processes influenced by testosterone-dependent and testosterone-independent genes, there were many

overlaps in the functional categories affected by the two groups. However, the genes involved in these functional categories were different in the testosterone and non-testosterone datasets implying that the sex steroid hormones and sex chromosomes work together to affect many processes by regulating different genes and separate aspects of the same pathways.

We also examined the effect of age on DNA methylation and found that the methylation status of thousands of genes was altered with increased age across all experimental groups. On the other hand, a large number of age-related genes were differentially methylated according to genotype or hormone treatment indicating that age-related effects on methylation can be sex-specific.

Altogether, our study emphasizes the importance of testosterone in regulating DNA methylation, which contributes to sexual differentiation. This study is unique because it challenges some of the views of organizational mechanisms of steroid action, which state that early exposure to testosterone establishes permanent sexually dimorphic effects that are maintained throughout life. Our results indicate that the effects of hormones are dynamic and the methylation patterns can change during the lifetime to make the sexes different or even similar. Most recent studies in the field of brain sexual differentiation focus on early hormone effects on methylation changes at particular CpG sites on specific promoters, especially on genes for steroid hormone receptors [23] but our project is to the best of our knowledge, the first comprehensive study of the organizational effects of hormones on a genome- wide scale.

It is important to note, however, that we are just beginning to understand the complexities of DNA methylation and many questions still remain regarding the functional significance of testosterone-induced epigenetic modifications. For instance, recent studies indicate that some

CpG sites might be more important than others for regulating gene expression, particularly if they reside within transcription factor response elements. Furthermore, DNA methylation might have other roles in addition to just modulating gene expression. Methylation might determine the use of alternative promoters controlling the expression of specific transcript variants [24] suggesting that testosterone-induced DNA modification could potentially provide a permissive condition for other neural processes that eventually lead to changes in the level, duration and/or isoforms of gene expression. Therefore, assessing the functional significance of testosterone-driven changes in methylation is of outmost importance.

Moreover, our study and most other studies that examine the role of DNA methylation in the brain, measure this mark using whole brain tissues, which are known to be composed of a highly heterogeneous cell population. Therefore, it is difficult to determine which cell types undergo methylation changes. Moreover, the magnitude of methylation change could be washed out when studying a highly heterogeneous cell population. Using flow cytometry [25], one can label and separate specific cell types (neurons or glial cells) from whole-tissue to determine where and in which cell populations methylation changes occur.

In this study, we have only investigated the link between testosterone and changes in DNA methylation. However, additional studies of other epigenetic markers (e.g., histone methylation, acetylation, 5-hydroxymethylcytosine, and microRNAs) across both sexes and different developmental time points can enable the identification of other candidate genes important in steroid hormone-induced organization of masculinized, defeminized, and feminized brain programs [26].

The possibility that differences in copy number variation and single nucleotide variations can impact sex differences in epigenetic modifications changes the way in which one studies brain sexual differentiation by providing insight into how hormones organize the brain. Longitudinal studies are also crucial to understanding whether the methylation changes are causally associated with the phenotype or are rather a consequence of it. These studies could help with identifying the timing of the change in methylation and aid in determining whether the methylation changes arose before the phenotype or if they were secondary to it [39]. Since our study only captures a snapshot of DNA methylation landscape, examining DNA methylation changes at additional time points can provide a comprehensive picture of the genome and further our understanding of how the epigenome is modified over time.

In conclusion, our methylation study has provided valuable insights into how early steroid hormone exposure can modify the neural DNA methylation landscape and how some of these effects emerge over time. It also suggests that the relationship between early epigenetic marks and adult pattern of methylation is complex. Given the potential role that epigenetic abnormalities might have in aging and neurological disorders [27, 28], the inducible malleability of DNA methylation in the brain by sex steroid hormones offers the possibility for developing novel strategies for sex-specific therapies.

SEX CHROMOSOME EFFECTS

Phenotypic differences between female and male mice are not just due to differences in gonadal hormone levels but can also be ascribed to different expression of X and Y genes. Despite strong evidence for the role that steroid hormone play in brain sexual differentiation, they are not the sole mediators of change. For instance, the Y chromosome, for example, carries a dominant testis-determining gene, *Sry*, that leads to the differentiation of testis from ovaries, which in turn establishes sex differences in the level of gonadal hormones and results in the differences between male and female cells. An example of the direct sex-specific effects of *Sry* on the brain can be seen in the dopamine-containing cells of the substantia nigra pars compacta (SNpc) that project to the striatum. These cells are the targets of Parkinson's disease. Men, who have a 1.5-fold higher incidence of this disease than woman, express *Sry* at these cells and when the expression of *Sry* is downregulated in the SNpc in adult male rats, tyrosine hydroxylase is substantially reduced in both the SNpc and striatum, causing a decline in motor function [29]. Interestingly, the SNpc and striatum work as well in females as in males, raising questions of whether a female-specific factor maintains tyrosine hydroxlyase levels and/or if another male-specific factor, such as testosterone, has a negative effect in this region that is offset by *Sry* expression. A new perspective emerging from the studies of sex chromosome genes and their effects on brain sexual differentiation is that sex-specific factors, such as sex chromosome genes or hormones may actually make the sexes more similar rather than different [30-32].

Another example of a sex chromosome gene that induces sex differences in phenotype is *Xist*. This gene is expressed from one of the two X chromosomes in females, causing inactivation of that chromosome, so that only one chromosome remains active transcriptionally [33, 34]. This

causes females to have similar effects as males due to a single X chromosome, but it also causes mosaicism in X chromosome gene effects.

Our work with regards to the four-core genotype (FCG) mouse model

Despite the strong evidence regarding the direct effects of sex chromosome genes, there have been very limited studies in this area, mostly because very few animal models can distinguish the effects of sex chromosome genes from the effects of gonadal hormones. One such model is the Four-Core Genotype (FCG) mouse model, which allows the direct manipulation of sex chromosomes and sex hormone levels independently from each other [31, 35, 36]. This model generates XX and XY mice with testes, or XX and XY mice with ovaries. This allows the simultaneous comparison of gonadal sex (comparing gonadal male and female phenotypes) and sex chromosome complement (comparing XX and XY mice of either phenotype). So far, very few sex differences in neural phenotypes have been studied in the FCG mouse model [37-47]. In most cases, the sex differences attributed to direct sex chromosome effects are also influenced by gonadal hormones, demanding further investigation of the interaction of sex-specific hormonal and sex chromosome effects.

Based on the evidence supporting the critical importance of direct genetic effects in producing brain sex differences, here we tested for sex chromosome effects on gene expression in the striatum of adult mice of the four core genotypes model. We were able to identify several novel genes whose expression was higher in XY vs. XX mice, regardless of the gonadal type. Interestingly, majority of these genes were located on the distal tip of the X chromosome near the mouse pseudoautosomal region and included genes such as *Msl3,* male-specific lethal-3, which is known to act within the dosage compensation complex in Drosophila [48].

XY and XX mice differ not only in the copy number (one vs. two copies) of X genes but they also differ in their parental imprint. Because the X chromosome of the XY mice is inherited from the father, whereas the X chromosome of the XX sisters is inherited from both parents and each imprint is expressed in about half of the cells because of X-inactivation. Therefore, here we speculated that these genes may be part of an imprinted gene cluster and sought to investigate the contribution of sex-specific imprinting to the observed sex differences. The significance of specific DNA methylation patterns is most clearly shown for the imprinted loci and evidence suggests that the allele-specific patterns of methylation are what govern allele-specific expression [49, 50]. Therefore, we began our studies by assessing the CpG methylation differences in the promoter region of one such genes (*Frmpd4*) in XX and XY animals of the FCG model. Our results revealed that there was a significant increase in mean DNA methylation levels of several CpG sites located near the *Frmpd4* transcription start site in the XX relative to XY animals, which was consistent with the observed lower expression of this gene in XX vs. XY animals.

Future work will be focused on a more detailed characterization of the *Frmpd4* gene for imprinting, and examining its temporal expression in the brain. *Frmpd4*, a novel PSD-95-interacting protein expressed throughout the mouse brain is involved in regulation of excitatory synapses and spine morphogenesis [51]. Indeed, an approximate 0.57-Mb duplication at Xp22.2 including a part of *Frmpd4* and *Msl3*, which is located distally to *Frmpd4* and has been detected in a Japanese patient with mental retardation and autism [52]. In a separate study, direct sequencing of 111 X-linked synaptic genes in individuals with autism spectrum disorder (n = 142; 122 males and 20 females) or schizophrenia (n = 143; 95 males and 48 females) have resulted in discovery of several promising non-synonymous rare variants in genes encoding

proteins involved in regulation of neurite outgrowth and other various synaptic functions such as *Frmpd4* [53].

One strategy for identifying novel X-linked imprinted genes and their downstream effects is to use genetically engineered mice with a single maternal or paternal X (X^mO and X^pO mice) [54, 55]. Comparison of XO mice with a maternal vs. paternal X will reveal whether *Frmpd4*, *Msl3* are paternally or maternally inherited X chromosome region with differences in epigenetic status and indicate if the differences in imprinting of these genes contribute to sex differences in comparisons of XX and XY mice. Characterization of X-linked imprinted genes at a molecular level is important for understanding the molecular basis of sexually dimorphic behavioral traits and gives us important clues in interpreting sex differences in mental disorders with a suspected developmental basis [56]. These genes can potentially produce sex differences independent of gonadal hormone effects. In fact, many neurodevelopmental related genes are encoded on the X chromosome and X-linked imprinting could potentially have large effects on sexually dimorphic neurobiology.

In addition, the sex chromosome effects reported here could also be caused by the sex differences in the number of nigrostriatal DA neurons, which is reported to display a sex difference influenced by a sex chromosome effect, in studies of dissociated cultures of embryonic midbrain cells [57]. Thereby, investigating whether the higher expression of Frmpd4 in XY mice is caused by the sex difference in Sry expression will also be an important future direction.

The present study was conducted mainly to examine the role of sex chromosome complement, not the role of adult gonadal hormones. In fact, we gonadectomized our adult mice

to remove the sex differences caused by the activational effects of gonadal hormones in adult mice. While the sex differences observed between XX and XY FCG mice are therefore not caused by the effects of circulating adult hormones, it remains likely that within sex group differences in gonadal secretions prior to gonadectomy could have established the sex chromosome effects observed. That idea is not strongly supported by the results, because when we directly compared striatal expression of *Frmpd4* in gonadectomized FCG adults that were gonadal males or females, we found no significant effect of gonadal type.

It is difficult to predict the functional consequences of the greater expression of these genes in XY than XX mice. Parkinson's disease, caused by loss of nigrostriatal neurons and striatal dopamine is more prevalent in men than women and sex chromosome linkage of susceptibility genes could help explain the sex difference in Parkinson's disease [58, 59] . *Frmpd4*, a gene encoded on the X chromosome, is involved in regulation of dendritic spine density [60]. In Parkinson's disease, striatal medium spiny neurons (MSN) lose their dendritic spines. Interestingly, dendritic spine density has been found to be sexually dimorphic in the striatum and changes in dendritic spine density have important implications for striatal neuronal function [61-63]. Thus, the present results facilitate further investigations of the contribution of these X chromosome genes to sex chromosome effects in the control of diverse functions of the striatum and provide insight into the molecular mechanisms underlying diseases that affect the striatum in as sex-specific manner.

Our work with regards to the Klinefelter Sex Trisomy Mouse model

In a separate project, we also tested the idea that the number of sex chromosomes could affect highly sexually dimorphic behaviors. For this work, we employed a novel mouse model of Klinefelter Syndrome to study one of the most highly sexually dimorphic behavioral characteristics, sexual partner preference. The majority of the existing animal models of Klinefelter Syndrome cannot dissociate the sex chromosome-dependent sexual differentiation of partner preference from hormonal effects [64-66]. Our KS mouse model represented a unique model in which sex chromosome effects and the interaction of sex chromosome and hormonal effects could be tested simultaneously.

Based on recent evidence of increased incidence of male sex preference and gender non-conformity in KS men [67-69], we tested whether XXY male mice exhibit a different partner preference behavior than their XY male littermates and indeed when we quantified the time that the KS animals spent with either an estrous female or an intact male, XXYM spent significantly less time with the stimulus female than XYM and there was a trend for an increased male preference in XXYM suggesting a gene dosage effect from the additional X chromosome on this highly sexually dimorphic behavioral phenotype.

Given that XXYM mice showed a more female-typical partner preference, we also sought to determine if their brain gene expression profile was also feminized. Our gene expression data of mouse KS brains showed that among the list of genes that displayed significant basal sex differences in adult striatum, a subset of genes displayed a feminized pattern of expression and the proportion of these genes were much higher than what would be expected

by chance. Known X-inactivation escapees also appeared in the list of feminized genes suggesting that that the process of X-inactivation occurs normally in XXYM in striatum.

To determine the effect of the additional X chromosome in XXYM on gene expression, we analyzed gene expression differences between XXYM and XYM. A large number of genes including autosomal genes were found to be differentially expressed between the two genotypes suggesting X and autosomal gene interactions and indicating genome-wide effects of the X chromosome dosage on autosomal gene expression. The differences in gene expression between XXYM and XYM can be attributed to the presence of the additional X chromosome in XXYM, the interaction of the additional X with other chromosomes (autosomes and/or the Y) or differences in hormonal levels during the critical periods. In order to differentiate between these effects and further refine our gene list, we next applied an expression filter that selected only the genes, within the XXYM vs. XYM gene list, displaying expression difference in the XYM animals, compared to the XXM mice. This expression filter yielded a final working list of candidate gene probes that were differentially regulated in both datasets. These genes are potentially influenced directly by the presence of the additional X chromosome and/or its interactions with autosomes. Furthermore, several genes were detected to be present only in the XXYM vs. XYM comparison group. The differential expression of these genes can most likely be explained by the interactions between X and Y chromosome and/or differences in testosterone levels between XXYM and XYM during the critical period. Genes that are unique to XXYM vs. XYM comparison serve as potential candidates for genes associated with the partner preference behavioral trait and other phenotypes unique to KS/XXYM.

When we performed Gene Ontology analysis on the set of genes that were differentially expressed between XXY and XYM, we found overrepresentation of immune-related functions.

Recent studies link the process of brain sexual differentiation to the immune system and implicate important roles for microglia, cytokines, and immune system signaling in the process of brain sexual differentiation. McCarthy et al. have recently shown the masculinizing effect of estradiol during the critical period is mediated via prostaglandin E_2 (PGE_2) and that microglial activation in response to estrogen during the critical period contributes to neural processes that are important for brain sexual differentiation (apoptosis, neurite outgrowth, and axonal guidance). Altogether these data along with our observations demonstrate the presence of a crosstalk between the immune and nervous system in the context of sex differences in the brain.

Finally, we showed that the molecular mechanisms underlying the KS phenotype can be attributed to a gene dosage effect from the additional X chromosome. Consistent with our FCG data, we were able to show that hormones are not the only determinants of brain sex differences and that sex chromosomes genes also contribute substantially to sexual differentiation.

Our data further supported the existence of an identifiable feminized expression signature in mice with KS and revealed potential candidate genes that could be used in future studies to help explain some of the neurobehavioral phenotypes of KS. However, we should keep in mind that the behavioral and gene expression differences that we observe between our XXYM and XYM could result from the differences in hormonal levels between the two genotypes prior to gonadectomy, which in turn could lead to differences in the organization of brain regions implicated in partner preference and subsequent feminization.

In future studies, it will be of great importance to measure gonadal hormone levels at different developmental stages to better distinguish between the direct effects of sex chromosome makeup and those of differing androgen levels. Furthermore, the fact that the

XXYM mice exhibit differences in sex preference for mice might be due to social, not sexual, cues. Additional experiments are definitely required to deconstruct the critical components of this behavior and discover whether social or sexual cues are being modified in XXY mice.

CONCLUDING REMARKS

Although, much is known about sex differences in brain morphology and physiology, we still lack a full understanding of the broad gene networks that regulate sexual differentiation across the brain. Therefore, the overarching goal here was to gain an integrated understanding of the biological basis of sex differences in the brain. This proposal aimed at finding candidate genes that related to sex differences in the striatum. The following conclusions can be drawn from the present work: (1) DNA methylation contributes to the organizational effects of steroid hormones on the brain; (2) Hormonal effects mediated through these epigenetic mechanisms likely condition sex differences in the brain gradually over time contributing to the view that steroids are slow mediators of sexual differentiation, with long onset and offset; (3) gonadal hormones are not the only proximate factors that operate directly on brain cells to induce sexual differentiation, but other signals, for instance those encoded by the sex chromosomes or autosomal gene products acting downstream from sex chromosome genes, can also have profound sex-differentiating effects on the brain; (4) sex-specific signals can also act as compensatory variables to reduce sex differences rather than induce them.

Although this study is an important step toward advancing our understanding of the factors that contribute to sex differences in brain and behavior, our findings are preliminary and future research needs to examine the interplay between morphological, physiological, and gene expression. It is not currently possible to directly connect the changes in the brain to a subsequent change in behavior. In my opinion, the future of the research in the field of brain sex

differences will mainly be focused on molecular and epigenetic studies that provide a circuit-level understanding of how multiple brain regions or cellular and molecular processes affect each other's physiology and development to govern sexual differentiation of brain and behavior.

REFERENCES

1. Voskuhl, R., *Sex differences in autoimmune diseases.* Biology of sex differences, 2011. **2**(1): p. 1.
2. Swaab, D.F. and M.A. Hofman, *Sexual differentiation of the human hypothalamus in relation to gender and sexual orientation.* Trends in neurosciences, 1995. **18**(6): p. 264-70.
3. Wald, C. and C. Wu, *Biomedical research. Of mice and women: the bias in animal models.* Science, 2010. **327**(5973): p. 1571-2.
4. *Putting gender on the agenda.* Nature, 2010. **465**(7299): p. 665.
5. Phoenix, C.H., et al., *Organizing action of prenatally administered testosterone propionate on the tissues mediating mating behavior in the female guinea pig.* Endocrinology, 1959. **65**: p. 369-82.
6. Handa, R.J., et al., *Gonadal steroid hormone receptors and sex differences in the hypothalamo-pituitary-adrenal axis.* Horm Behav, 1994. **28**(4): p. 464-76.
7. Shors, T.J. and G. Miesegaes, *Testosterone in utero and at birth dictates how stressful experience will affect learning in adulthood.* Proc Natl Acad Sci U S A, 2002. **99**(21): p. 13955-60.
8. Craft, R.M., J.S. Mogil, and A.M. Aloisi, *Sex differences in pain and analgesia: the role of gonadal hormones.* Eur J Pain, 2004. **8**(5): p. 397-411.
9. Breedlove, S.M. and A.P. Arnold, *Hormonal control of a developing neuromuscular system. II. Sensitive periods for the androgen-induced masculinization of the rat spinal nucleus of the bulbocavernosus.* J Neurosci, 1983. **3**(2): p. 424-32.

10. Breedlove, S.M. and A.P. Arnold, *Hormonal control of a developing neuromuscular system. I. Complete Demasculinization of the male rat spinal nucleus of the bulbocavernosus using the anti-androgen flutamide.* J Neurosci, 1983. **3**(2): p. 417-23.

11. Juraska, J.M., *Sex differences in "cognitive" regions of the rat brain.* Psychoneuroendocrinology, 1991. **16**(1-3): p. 105-9.

12. Juraska, J.M., *Neural plasticity and the development of sex differences.* Annu Rev Sex Res, 1998. **9**: p. 20-38.

13. Beato, M., *Gene regulation by steroid hormones.* Cell, 1989. **56**(3): p. 335-44.

14. Beato, M. and J. Klug, *Steroid hormone receptors: an update.* Hum Reprod Update, 2000. **6**(3): p. 225-36.

15. Woolley, C.S., *Acute effects of estrogen on neuronal physiology.* Annu Rev Pharmacol Toxicol, 2007. **47**: p. 657-80.

16. Forger, N.G., et al., *Deletion of Bax eliminates sex differences in the mouse forebrain.* Proc Natl Acad Sci U S A, 2004. **101**(37): p. 13666-71.

17. Waters, E.M. and R.B. Simerly, *Estrogen induces caspase-dependent cell death during hypothalamic development.* J Neurosci, 2009. **29**(31): p. 9714-8.

18. Krishnan, S., et al., *Central role of TRAF-interacting protein in a new model of brain sexual differentiation.* Proc Natl Acad Sci U S A, 2009. **106**(39): p. 16692-7.

19. Zhang, J.M., et al., *Impact of sex and hormones on new cells in the developing rat hippocampus: a novel source of sex dimorphism?* Eur J Neurosci, 2008. **27**(4): p. 791-800.

20. de Vries, G.J., et al., *Sexual differentiation of vasopressin innervation of the brain: cell death versus phenotypic differentiation.* Endocrinology, 2008. **149**(9): p. 4632-7.

21. Wright, C.L., S.R. Burks, and M.M. McCarthy, *Identification of prostaglandin E2 receptors mediating perinatal masculinization of adult sex behavior and neuroanatomical correlates.* Dev Neurobiol, 2008. **68**(12): p. 1406-19.

22. Dunn, G.A., C.P. Morgan, and T.L. Bale, *Sex-specificity in transgenerational epigenetic programming.* Horm Behav, 2011. **59**(3): p. 290-5.

23. Schwarz, J.M., B.M. Nugent, and M.M. McCarthy, *Developmental and hormone-induced epigenetic changes to estrogen and progesterone receptor genes in brain are dynamic across the life span.* Endocrinology, 2010. **151**(10): p. 4871-81.

24. Monje, L., et al., *Neonatal exposure to bisphenol A modifies the abundance of estrogen receptor alpha transcripts with alternative 5'-untranslated regions in the female rat preoptic area.* The Journal of endocrinology, 2007. **194**(1): p. 201-12.

25. Jiang, Y., et al., *Isolation of neuronal chromatin from brain tissue.* BMC neuroscience, 2008. **9**: p. 42.

26. Lenz, K.M., B.M. Nugent, and M.M. McCarthy, *Sexual differentiation of the rodent brain: dogma and beyond.* Frontiers in neuroscience, 2012. **6**: p. 26.

27. Ma, D.K., et al., *Epigenetic choreographers of neurogenesis in the adult mammalian brain.* Nature neuroscience, 2010. **13**(11): p. 1338-44.

28. Tsankova, N., et al., *Epigenetic regulation in psychiatric disorders.* Nature reviews. Neuroscience, 2007. **8**(5): p. 355-67.

29. Dewing, P., et al., *Direct regulation of adult brain function by the male-specific factor SRY.* Current biology : CB, 2006. **16**(4): p. 415-20.

30. De Vries, G.J., *Minireview: Sex differences in adult and developing brains: compensation, compensation, compensation.* Endocrinology, 2004. **145**(3): p. 1063-8.

31. Arnold, A.P. and X. Chen, *What does the "four core genotypes" mouse model tell us about sex differences in the brain and other tissues?* Frontiers in Neuroendocrinology, 2009. **30**(1): p. 1-9.

32. McCarthy, M.M. and A.T. Konkle, *When is a sex difference not a sex difference?* Frontiers in Neuroendocrinology, 2005. **26**(2): p. 85-102.

33. Chang, S.C., et al., *Mechanisms of X-chromosome inactivation.* Frontiers in bioscience : a journal and virtual library, 2006. **11**: p. 852-66.

34. Migeon, B.R., *Why females are mosaics, X-chromosome inactivation, and sex differences in disease.* Gender medicine, 2007. **4**(2): p. 97-105.

35. De Vries, G.J., et al., *A model system for study of sex chromosome effects on sexually dimorphic neural and behavioral traits.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2002. **22**(20): p. 9005-14.

36. Arnold, A.P., et al., *Minireview: Sex chromosomes and brain sexual differentiation.* Endocrinology, 2004. **145**(3): p. 1057-62.

37. Gatewood, J.D., et al., *Sex chromosome complement and gonadal sex influence aggressive and parental behaviors in mice.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2006. **26**(8): p. 2335-42.

38. Gioiosa, L., et al., *Sex chromosome complement affects nociception and analgesia in newborn mice.* The journal of pain : official journal of the American Pain Society, 2008. **9**(10): p. 962-9.

39. Gioiosa, L., et al., *Sex chromosome complement affects nociception in tests of acute and chronic exposure to morphine in mice.* Hormones and behavior, 2008. **53**(1): p. 124-30.

40. Quinn, J.J., et al., *Sex chromosome complement regulates habit formation.* Nature neuroscience, 2007. **10**(11): p. 1398-400.

41. Barker, J.M., et al., *Dissociation of genetic and hormonal influences on sex differences in alcoholism-related behaviors.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2010. **30**(27): p. 9140-4.

42. Chen, X., et al., *Sex difference in neural tube defects in p53-null mice is caused by differences in the complement of X not Y genes.* Developmental neurobiology, 2008. **68**(2): p. 265-73.

43. Smith-Bouvier, D.L., et al., *A role for sex chromosome complement in the female bias in autoimmune disease.* The Journal of experimental medicine, 2008. **205**(5): p. 1099-108.

44. Cox, K.H. and E.F. Rissman, *Sex differences in juvenile mouse social behavior are influenced by sex chromosomes and social context.* Genes, brain, and behavior, 2011. **10**(4): p. 465-72.

45. McPhie-Lalmansingh, A.A., et al., *Sex chromosome complement affects social interactions in mice.* Hormones and behavior, 2008. **54**(4): p. 565-70.

46. Chen, X., W. Grisham, and A.P. Arnold, *X chromosome number causes sex differences in gene expression in adult mouse striatum.* The European journal of neuroscience, 2009. **29**(4): p. 768-76.

47. Abel, J.M., D.M. Witt, and E.F. Rissman, *Sex differences in the cerebellum and frontal cortex: roles of estrogen receptor alpha and sex chromosome genes.* Neuroendocrinology, 2011. **93**(4): p. 230-40.

48. Lucchesi, J.C., *Dosage compensation in flies and worms: the ups and downs of X-chromosome regulation.* Current opinion in genetics & development, 1998. **8**(2): p. 179-84.

49. Yoon, B.J., et al., *Regulation of DNA methylation of Rasgrf1.* Nat Genet, 2002. **30**(1): p. 92-6.

50. Li, E., C. Beard, and R. Jaenisch, *Role for DNA methylation in genomic imprinting.* Nature, 1993. **366**(6453): p. 362-5.

51. Lee, H.W., et al., *Preso, a novel PSD-95-interacting FERM and PDZ domain protein that regulates dendritic spine morphogenesis.* The Journal of neuroscience : the official journal of the Society for Neuroscience, 2008. **28**(53): p. 14546-56.

52. Honda, S., et al., *Copy-number variations on the X chromosome in Japanese patients with mental retardation detected by array-based comparative genomic hybridization analysis.* Journal of human genetics, 2010. **55**(9): p. 590-9.

53. Piton, A., et al., *Systematic resequencing of X-chromosome synaptic genes in autism spectrum disorder and schizophrenia.* Molecular psychiatry, 2011. **16**(8): p. 867-80.

54. Evans, E.P. and R.J. Phillips, *Inversion heterozygosity and the origin of XO daughters of Bpa/+female mice.* Nature, 1975. **256**(5512): p. 40-1.

55. Burgoyne, P.S. and E.P. Evans, *A high frequency of XO offspring from X(Paf)Y* male mice: evidence that the Paf mutation involves an inversion spanning the X PAR boundary.* Cytogenetics and cell genetics, 2000. **91**(1-4): p. 57-61.

56. Davies, W., et al., *X-linked imprinting: effects on brain and behaviour.* BioEssays : news and reviews in molecular, cellular and developmental biology, 2006. **28**(1): p. 35-44.

57. Carruth, L.L., I. Reisert, and A.P. Arnold, *Sex chromosome genes directly affect brain sexual differentiation.* Nat Neurosci, 2002. **5**(10): p. 933-4.

58. Pankratz, N., et al., *Genome-wide linkage analysis and evidence of gene-by-gene interactions in a sample of 362 multiplex Parkinson disease families.* Hum Mol Genet, 2003. **12**(20): p. 2599-608.

59. Maraganore, D.M., et al., *High-resolution whole-genome association study of Parkinson disease.* Am J Hum Genet, 2005. **77**(5): p. 685-93.

60. Lee, H.W., et al., *Preso, a novel PSD-95-interacting FERM and PDZ domain protein that regulates dendritic spine morphogenesis.* J Neurosci, 2008. **28**(53): p. 14546-56.

61. Forlano, P.M. and C.S. Woolley, *Quantitative analysis of pre- and postsynaptic sex differences in the nucleus accumbens.* J Comp Neurol, 2010. **518**(8): p. 1330-48.

62. Stephens, B., et al., *Evidence of a breakdown of corticostriatal connections in Parkinson's disease.* Neuroscience, 2005. **132**(3): p. 741-54.

63. Zaja-Milatovic, S., et al., *Dendritic degeneration in neostriatal medium spiny neurons in Parkinson disease.* Neurology, 2005. **64**(3): p. 545-7.

64. Liu, P.Y., et al., *Genetic, hormonal, and metabolomic influences on social behavior and sex preference of XXY mice.* Am J Physiol Endocrinol Metab, 2010. **299**(3): p. E446-55.

65. Swerdloff, R.S., et al., *Mouse model for men with klinefelter syndrome: a multifaceted fit for a complex disorder.* Acta paediatrica, 2011. **100**(6): p. 892-9.

66. Lue, Y., et al., *XXY mice exhibit gonadal and behavioral phenotypes similar to Klinefelter syndrome.* Endocrinology, 2005. **146**(9): p. 4148-54.

67. Bancroft, J., D. Axworthy, and S. Ratcliffe, *The personality and psycho-sexual development of boys with 47 XXY chromosome constitution.* J Child Psychol Psychiatry, 1982. **23**(2): p. 169-80.

68. Theilgaard, A., *A psychological study of the personalities of XYY- and XXY-men.* Acta psychiatrica Scandinavica. Supplementum, 1984. **315**: p. 1-133.

69. Schiavi, R.C., et al., *Sex chromosome anomalies, hormones, and sexuality.* Arch Gen Psychiatry, 1988. **45**(1): p. 19-24.