Title
The Effects of Chromosomal Composition and Hormonal Influences on Shaping Sex Differences in the Developing Mammalian Brain

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Publication Date
2017

UNIVERSITY OF CALIFORNIA

Los Angeles

The Effects of Chromosomal Composition and Hormonal Influences on Shaping Sex Differences in the Developing Mammalian Brain

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Human Genetics

by

Matthew Scott Bramble

2017

ABSTRACT OF THE DISSERTATION

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Matthew Scott Bramble

Doctor of Philosophy in Human Genetics

University of California, Los Angeles, 2017

Professor Eric J.N. Vilain, Chair

The mechanisms by which sex differences in the mammalian brain arise are poorly understood, but are influenced by a combination of underlying genetic differences and gonadal hormone exposure. Using a mouse embryonic neural stem cell (eNSC) model to understand early events contributing to sexually dimorphic brain development, we identified novel interactions between chromosomal sex and hormonal exposure that are instrumental to early brain sex differences. RNA-sequencing identified 103 transcripts that were differentially expressed between XX and XY eNSCs at baseline (FDR=0.10). Treatment with testosterone-propionate (TP) reveals sex-specific gene expression changes, causing 2854 and 792 transcripts to become differentially expressed on XX and XY genetic backgrounds respectively. These findings indicate that testosterone exposure on XX cells have a more robust effect with regards to altering gene expression. It was also found that by exposing XX eNSCs to TP 42% (43/103) of the original 103 basal sex differences that existed became masculinized and shifted towards a XY typical gene expression pattern. We also determined that 25% (26/103) of basal sex differences

were actually feminized in an XY background post-TP. These alteration in gene expression post-TP exposure were determine to require functional androgen receptor (AR), as AR knockout (ARKO) eNSCs did not show differential expression of select genes in the presence of TP. Within the TP responsive transcripts, there was enrichment for genes which function as epigenetic regulators that affect both histone modifications and DNA methylation patterning, specifically Tet proteins, known to function as a DNA demethylases. We observed that TP caused a global decrease in 5-methylcytosine abundance in both sexes, a transmissible effect that was maintained in cellular progeny. Like gene expression, alteration in DNA methylation function downstream of AR activation, with ARKO lines showing no significant androgen induced DNA de-methylation. Additionally, we determined that TP was associated with residue-specific alterations in acetylation of histone tails. Collectively, these findings highlight an unknown component of androgen action on cells within the developmental CNS, and contribute to a novel mechanism of action by which early hormonal organization is initiated and maintained.

The dissertation of Matthew Scott Bramble is approved.

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ACKNOWLEDGEMENTS

I would like to first acknowledge Eric Vilain, for his mentorship, strong support and encouraging my development as an independent scientist and free thinker. His mentorship has greatly contributed to both my academic and personal success over the course of these past 5 years. Much of my work would have been near impossible without the support of my dedicated undergraduate research team. With that, I thank Lara Roach, Jason Gosschalk and Allen Lipson for their great contribution to this thesis and the various publications that have resulted along the way.

I owe an exceptionally huge debt of gratitude to Neerja Vashist, who has worked with me from the beginning of graduate school and has always stuck by my side, even in the bleakest of research times. Without her contributions, support and superb scientific capabilities, I am certain that my thesis would have been far inferior. I also thank Nantao Ying for patiently dealing with the many stresses and ups and downs that have occurred along this long Ph.D. road. I am also greatly appreciative of the esteemed members of my thesis committee, Katrina Dipple, Julian Martinez-Agosto and Art Arnold. Their guidance, advice and constructive criticisms over the course of graduate school have significantly contributed to this work, as well as my ability to coherently defend my ideas with strong scientific arguments. Much of my work would have been near impossible if not for my many collaborators and co-authors that have provided their expertise and assistance on various papers and projects over these past five years. Additionally, I thank Hayk Barseghyan, Francisco Sanchez, Tuck Ngun, Mehmet Keles and Valerie Arboleda for their support, friendship, advice and making this research and experience truly enjoyable along the way!

Chapter 1 is a published review from a special issue focused on sex-differences, in the Journal of Neuroscience Research with the following citation: Bramble, M. S., Lipson, A., Vashist, N. and Vilain, E. (2017), Effects of chromosomal sex and hormonal influences on shaping sex differences in brain and behavior: Lessons from cases of disorders of sex development. Journal of Neuroscience Research, 95: 65–74. doi:10.1002/jnr.23832

Chapter 2 is another prepared review manuscript that has been submitted and will contain the following citation: Bramble MS, Vashist N, Lipson A, Vilain E: Sex-Differences and Gonadal Hormone Response in Cultured Mammalian Neural Stem Cells: A Contributing Element to Hormonal Brain Organization and Neurodevelopment? 2017.

Chapters 3 and 4 contain unpublished data, along with excerpts from a publication with the following citation: Bramble MS, Roach L, Lipson A, Vashist N, Eskin A, Ngun T, Gosschalk JE, Klein S, Barseghyan H, Arboleda VA, Vilain E. 2016. Sex-Specific Effects of Testosterone on the Sexually Dimorphic Transcriptome and Epigenome of Embryonic Neural Stem/Progenitor Cells. Scientific Reports 6:36916.

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Publications----------

1) **Bramble MS**, Goldstein EH, Lipson A, Ngun T, Eskin A, Gosschalk JE, Roach L, Vashist N, Barseghyan H, Lee E, Arboleda VA, Vaiman D, Yuksel Z, Fellous M, Vilain E. 2016. **A novel follicle-stimulating hormone receptor mutation causing primary ovarian failure: a fertility application of whole exome sequencing**. Human reproduction (Oxford, England) 31(4):905-914.

2) **Bramble, M. S.**, Lipson, A., Vashist, N. and Vilain, E. (2017), **Effects of chromosomal sex and hormonal influences on shaping sex differences in brain and behavior: Lessons from cases of disorders of sex development**. Journal of Neuroscience Research, 95: 65–74. doi:10.1002/jnr.23832

3) **Bramble MS**, Roach L, Lipson A, Vashist N, Eskin A, Ngun T, Gosschalk JE, Klein S, Barseghyan H, Arboleda VA, Vilain E. 2016. **Sex-Specific Effects of Testosterone on**

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6) Barseghyan H, Symon A, Zadikyan M, Almalvez M, Segura E, Eskin A, **Bramble MS**, Arboleda VA, Baxter R, Nelson SF *et al*: **Identification of Novel Candidate Genes for 46,XY Disorders of Sex Development (DSD) using C57BL/6J-YPOS Mouse Model**. 2017. (Under Revew)

7) Barseghyan H, Tang W, Wang RT, Almalvez M, Segura E, **Bramble MS**, Lipson A, Douine ED, Lee H, Delot E, Nelson SF, Vilain E. 2017. **Nex-Generation Mapping (NGM): A Novel Approach for Clinical Genetic Diagnosis of Structral Variants.** (Under Review)

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Chapter 1: Broad Introduction

The Effects of Chromosomal Sex and Hormonal Influences on Shaping Sex Differences in Brain and Behavior: Lessons from Cases of Disorders of Sex Development

Abstract: Sex differences in brain development and postnatal behavior are largely determined by genetic sex and *in utero* gonadal hormone secretions. In humans however, determining the weight that each of these factors contribute remains a challenge, as social influences should also be considered. Cases of disorders of sex development (DSD) provide unique insight to infer how mutations in genes responsible for gonadal formation can perturb the subsequent developmental hormonal milieu and elicit changes in normal human brain maturation. Specific forms of DSDs such as complete androgen insensitivity syndrome (CAIS), congenital adrenal hyperplasia (CAH), and 5-alpha-reductase deficiency syndrome have variable effects between males and females, as the developmental outcomes of such conditions are largely dependent on sex chromosome composition. Medical and psychological works focused on CAH, CAIS and 5-α-reductase deficiency have helped form the foundation for understanding the roles of genetic and hormonal factors necessary for guiding human brain development. Here we highlight how the three aforementioned DSDs contribute to brain and behavioral phenotypes that can uniquely affect 46, XY and 46, XX in dramatically different fashions.

Significance Statement: Sex differences in the brain and behavior of humans have been identified on numerus measures. The cause of these sex differences has been associated with chromosomal constitution and circulating hormones, such as estrogen and testosterone. Research on individuals with DSD has improved the scientific community's understanding of the potential contributions of the X and Y chromosome as well as the role of gonadal hormone secretions in generating these differences. This review highlights research that has expanded our understanding of the origins of specific sex differences observed in humans such as sexual orientation, gender identity and cognitive performance.

Introduction: Research investigating the role that hormones and genetics contribute towards guiding sexual differentiation and behavior in rodents has been extensively reviewed elsewhere [1, 2]. Strong evidence from such studies suggests that testosterone, either directly or indirectly, is responsible for organizing male-typical features, including gender role behavior, sexual orientation and perhaps gender identity. Evidence provided from the rodent model has laid the groundwork for our understanding of how *in utero* hormonal milieu and chromosomal composition influence brain development and contribute to generating observed sex differences. Unfortunately, similar research in humans is lacking in adequate depth from a biological standpoint. Investigating sex differences in human brain structure and cognitive development is challenging, as humans are a socially influenced species, making it difficult to tease apart the effects of environmental influences from biological contributions. Studying unique cases of DSDs where chromosomal composition and hormonal alterations are present has greatly improved our understanding of biological factors that contribute to sex differences within the human brain, from cognitive to structural. Here, we review and highlight how specific disorders of sex development have improved our understanding of the origins of certain sex differences within the brain, including gender-role and identity, sexual orientation, cognitive abilities as well as actual structural variations, that correspond with gender differences in humans.

Etiology of Congenital Adrenal Hyperplasia, Complete Androgen Insensitivity Syndrome, and 5α-Reductase Deficiency:

Congenital Adrenal Hyperplasia: CAH is the most common condition that contributes to incidences of ambiguous genitalia in 46, XX newborns. It is a recessive autosomal disorder that affects approximately 1 in 15,000 live births worldwide [3, 4]. 95% of known CAH cases result

from mutations in the CYP21A2 gene on chromosome 6, encoding the 21-hydroxylase (21-OH) enzyme [4]. Mutations in 21-OH generates two types of classical congenital adrenal hyperplasia; simple virilizing and the more severe form known as salt-wasting. By virtue of the mutation, many steroid hormone precursors within the adrenals become elevated due to improper metabolism into the necessary glucocorticoids, namely cortisol [5]. In addition to causing a cortisol and aldosterone deficiency, some of the hormone precursors are shuttled into the androgen synthesis pathway, leading to increased levels of testosterone in both XX and XY fetuses [6, 7]. As a result of CAH, female patients are often born with various degrees of genital virilization, typically consisting of a partially fused rugated labia majora, a common urogenital sinus and clitoromegaly—providing an early diagnostic sign of CAH in XX patients [7, 8]. 46, XY patients have minor to unnoticeable physical signs of the condition at birth, with the exception of some cases of hyperpigmentation of the genitals, and above average penile growth. The subtle nature of the clinical presentations in males results in a lower frequency of initial CAH diagnosis, and subsequently higher death rates for salt wasting cases [3, 8]. In regards to treatment, both XX and XY CAH patients are supplemented with life-long mineralocorticoid and glucocorticoid replacement therapies in the forms of fludrocortisone and hydrocortisone, to supplement their cortisol deficiency and reduce the over production of hormone precursors [4]. Prevention of female genital virilization in the unborn fetus can be achieved by administering the steroid dexamethasone to the pregnant mother. This practice is quite controversial as the therapy must begin before fetus sex is determined, thereby exposing 7 out of 8 unaffected fetuses to DEX treatment, with potential unknown side effects on the developing brain [9] (**Fig.1**).

Complete Androgen Insensitivity Syndrome: Complete androgen insensitivity syndrome (CAIS) is the most common disorder of sex development causing sex reversal exclusively on a 46, XY chromosomal background. The prevalence of this disorder among live births worldwide is estimated to range between 1:20,000 and 1:99,000 [10, 11]. 95% of all CAIS cases can be explained by inactivating mutations of the androgen receptor (AR) gene [11, 12]. Over 500 mutations in AR have been associated with CAIS, the majority occurring either within the DNA-binding domain (DBD) or the ligand-binding domain (LBD), rendering the protein inactive [13-15] . 46, XY patients born with CAIS are universally assigned as females at birth due to the outward female phenotypic appearance of the genitalia. The diagnosis often occurs during pubertal years where patients present with primary amenorrhea, due to lack of internal female structures. Individuals with CAIS have a blind-ending vagina, absent ovaries, inguinal testes and no uterus, due to the regression of Müllerian structures via AMH secretion by the testes [11]. If inguinal testes are not removed before puberty, there is often normal breast development due to the high levels of circulating testosterone which is locally aromatized into active estrogens, stimulating breast growth. If gonads have been resected prior to puberty, estradiol supplementation is often administered to bring the 46, XY CAIS patients into the normal female range of the given hormone [11] (**Fig. 1**).

5-α-Reductase Deficiency: First identified in 1974, this DSD is an autosomal recessive condition caused by mutations within the SRD5A2 gene located on chromosome 2, and manifests as phenotypically hypo-masculinized genitals in 46, XY individuals [16, 17]. This is a very rare disorder, with most documented cases occurring in areas of the world with high rates of consanguine mating, including the Dominican Republic, the islands of New Guinea and areas of

the Middle East such as Turkey and Iraq [18-22]. SRD5A2 encodes a steroid 5-α-reductase-2 enzyme, which is responsible for converting testosterone (T) into the more potent and developmentally necessary form, dihydrotestosterone (DHT). DHT is critical for the masculinization of genital tissue, and aids in proper penile growth and prostate development [16, 23]. Due to the improper conversion of T into DHT, 46, XY individuals born with this condition present with undescended inguinal testis, normal internal male structures and a reduction in prostate size [23]. Externally however, this mutation leads to various degrees of genital ambiguity, appearing as complete female to nearly that of typical male infants. Those with genital ambiguity often present with a bifid scrotum, enlarged clitoris, blind vagina pouch, and various severities of hypospadias [23]. During teenage years the undescended testis begin to secrete high levels of T and trigger male puberty, characterized by the deepening of the voice, increased muscle mass and height. In addition, there can be a dramatic increase in the growth of the phallus and occasional descent of the testes into the scrotal region [23] (**Fig. 1**).

Sexual Behaviors and Gender-Identity:

Two of the largest measurable sex differences observed between humans are gender identity and sexual orientation [24]. Deciphering what brings about these differences is a subject of much debate; however, many in the scientific community attribute them as being mediated in part by sex chromosomes and more directly by gonadal hormone secretions *in utero*. The theory of brain organization arose from mid-20th century works, where it was observed that administering testosterone to female rodents during a critical period for fetal brain organization brought about lifelong sexual and behavioral shifts [25]. Work in humans addressing this subject

is much more limited compared to studies in other mammals. Cases of DSDs provide us with unique insights into determining if and how male sex hormones are capable of establishing these sex differences with regards to sexual orientation and gender identity.

A study conducted by the Williams Institute of UCLA using large data sets from western societies, found that roughly 3.5% of the general population within the United States identified as having non-heterosexual orientation, and roughly 8.2% were determined as having experienced same-sex encounters [26]. Numerous studies have assessed the prevalence of non-heterosexual behaviors amongst 46, XX women with CAH from various regions of the world. This condition is the closest mimic of rodent studies that address the role of early androgen exposure and variations seen in adult behavior. Generally speaking, it has been found in the majority of studies that non-heterosexual behaviors are increased in women with CAH, typically compared to their unaffected female relatives or appropriate controls. In a study conducted by Zucker et al. focusing on the most severe salt-wasting form of CAH, as well as the milder simple virilizing form, it was found that around 27% of women with CAH had bisexual/homosexual sexual fantasies. Only 3.3% of individuals in the study had actual non-heterosexual partners which was not significantly different than controls [27]. A more recent study out of France found similar findings as Zucker, where they identified within their CAH cohort (individuals with varying degrees of genital virilization as measured on the Prader scale) that 20% exhibited bisexual and homosexual fantasies and only 5.8% had opposite sexual partners [28] . Non-heterosexual behaviors and fantasies amongst women with CAH have been identified at varying percentage levels, as highlighted in an exhaustive account of such studies compiled by Meyer-Bahlburg and colleagues [29]. In contrast however, other studies conducted on the topic have also found no differences in sexual orientation [30, 31]; however, some critics of these works

have determined that the sample size in certain studies were too underpowered to detect a difference [29]. Using a large CAH cohort, it was again revealed that women with CAH show more homosexual and bisexual behaviors, as measured by their sexual fantasies and actual sex partners. The study also successfully demonstrated that the level of non-heterosexual tendencies increased with the severity of the condition, a correlation associated with the level of *in utero* androgen exposure. Within the study cohort it was found that 47% of patients with the salt-wasting form of the condition experienced bisexual or homosexual fantasies, 33% for those with the simple virilizing form, and surprisingly 24% for those with the non-classical form of CAH [29]. Biologically, it appears that *in utero* androgen exposure on an XX background most significantly affects sexual fantasies in women with CAH more so than directly altering their actual sexual orientation as measured by sexual partners, which may be influenced by additional constraints. These findings provide a strong argument for a biological influences on eliciting such outcomes; however, these studies do not eliminate the possibility of social and other psychological contributions for these observations, as reviewed by Rebecca Jordan-Young [32].

In addition to sexual orientation, gender identity (one's internal sense of being male or female, independent of gonadal sex) has also been a topic of investigation in cases of DSD. Among the compiled studies that have focused on 46, XX CAH and gender identity, it was found that the vast majority, (95%) of patients who were assigned to the female gender were satisfied and non-dysphoric in adulthood [33]. However 5% of CAH females exhibited gender dysphoria and/or identified as the male gender as reviewed by Dessens and others [33]. This 5% of dysphoria in CAH females is far above the estimated average of 0.3% of adults who identify as transgender within western societies, specifically the U.S. [26]. In instances where 46, XX CAH were assigned to the male gender at birth, a small sampled study showed that 12% of cases

experienced gender dysphoria and associated themselves with the female gender [33]. However, later studies assessing outcomes in severely virilized 46, XX cases who were assigned as males at birth drew different conclusions about satisfaction of male-gender assignment and identity [34]. In a small study population of twelve 46, XX CAH cases who were initially assigned as males, it was found that ten persisted in the assigned gender and lived male-typical and satisfying lives. Two of the male-assigned individuals within the study that were adolescently re-assigned to the female gender, subsequently re-assigned themselves back to the male gender in adulthood [34, 35]. Unlike results found in studies examining sexual orientation, dysphoria in gender identity was neither correlated with the severity of the CAH condition, nor the degree of genital virilization, demonstrating that genital appearance at birth is not the best predictor for gender outcomes in cases of 46, XX DSD [36]. Collectively, these data demonstrate that we have yet to fully understand gender identity determinants in CAH individuals, and the general population for that matter, from a strictly biological perspective. It is however apparent that androgen exposure on XX genetic backgrounds can increase instances of dissatisfactions of ones gender assignment at rates far above estimated percentages found within general populations.

Some of the most convincing evidence linking testosterone to establishing male gender identity and sexual preference comes from work focused on individuals with complete androgen insensitivity syndrome and those with 5-α-reductase deficiencies. When assessing sexual orientation in cases of CAIS, it has been shown that 46, XY women are almost always exclusively heterosexual, preferring male sex partners, in addition to experiencing heterosexual fantasies and interests [37, 38]. During adolescence years CAIS women reported feeling 100 percent heterosexual and a minority (n=1) in a particular study stated same-sex attraction, but only in adulthood. This occurrence as the authors note may be influenced by other causes as

opposed to their inability to respond to androgens during development [37]. In contrast, recent literature has begun to challenge this strongly established view of nearly exclusive heterosexual behaviors in women with CAIS. In a recent report by Brunner and colleagues, it was found that 46, XY women when compared to other females with infertility conditions showed increased tendencies of non-heterosexual preference and behavior—evidence that is out of line with previously established views of fully heterosexual outcomes in women with CAIS. [39]. These findings, while limited to a small sample size (n=11 CAIS), raise an interesting topic for further investigation that could identify additional influences that guide sexual preference and perhaps identity in 46, XY women, aside from their inability to respond to male-sex hormone.

Using a more scientifically unbiased approach to assess sexual response, and perhaps orientation in women with CAIS, a recent study focused on neural activation in response to viewing sexual images. Supporting the notion of predominantly heterosexual feelings in CAIS, it was found that women with this condition more closely resembled 46, XX heterosexual females than 46, XY males [40]. Functional MRI (fMRI) scans revealed that both 46, XY women with CAIS and 46, XX women had decreased amygdala activation when viewing nude male images or couples engaging in coitus. The results of this study were the first to establish that the brains of CAIS women more functionally resembled that of a typical female, further indicating that testosterone responsiveness (and not the Y chromosome), is the most likely driver for establishing gynephilic preference, and androphilic-typical brain activation towards sexual stimuli in humans [40].

In regards to gender identity, 46, XY women with CAIS almost always report feeling comfortable in their female assignment, as shown by their typical female responses to assessments measuring psychosocial traits [38]. A thorough review of CAIS studies identified

that out of the 156 individuals assessed in the research, there were no instances of male-gender identity reported [41]. These findings add strength to the biological notion that hormonal response influences male-gender identity more so than chromosomal composition. Once again however, recent reports have begun to suggest that this theory is not as concrete as previously thought. A growing body of literature and case reports exists which have identified gender-dysphoria and cross-gender feelings in CAIS individuals at rates higher than have been established [39, 42, 43]. The role of social and initial medical decisions associated with CAIS cases may be a contributing factor in these results because as stated by Tom Mazur, "The best predictor of adult gender identity in CAIS, PAIS (partial androgen insensitivity syndrome) and micropenis is initial gender assignment" [41].

In addition to CAIS, research focused on the understudied and rare 5-α-reductase deficiency syndrome has also helped expand the role of testosterone in establishing sex differences in regards to sexual orientation and gender identity in humans. Individuals affected with this condition are typically reared as girls; however, during the pubertal years when testosterone secretions from the testes are elevated and male puberty ensues, the vast majority change initial gender assignment [22]. This outcome in persons with 5-α-reductase deficiency appears to be worldwide, and not necessarily a product of specific social influences, as gender-role and identity changes have been reported in cases from the Mediterranean, Middle East, Caribbean, Africa and New Guinea [22, 44, 45]. After change in gender role and gender identity these 46, XY individuals typically lead heterosexual lives, preferring female partners, despite having been initially reared as female themselves [22]. This gender switch in individuals during male puberty raises the notion that despite insufficient DHT production necessary for typical male genitalia development, the organizational effects on the brain were established and able to

influence male-gender role and eventually their identity, which seem to become solidified upon pubertal testosterone surges. 5-α-reductase deficiency syndrome is an unusual circumstance where teasing apart the effects of environmental influence and biology becomes extremely difficult. The activational effects of testosterone at puberty coupled with likely *in utero* organization do appear to be factors in establishing the change in gender role and identity. However, the physical change that also ensues during the transition process cannot be ruled out as another contributing factor for the observed change.

Highlight of Cognitive Sex Differences and Interpretation from Cases of DSD:

Sex differences in regards to cognitive traits have been extensively studied by numerous groups and have identified several measures where males and females show large deviations in mental task abilities. The origin of these sex differences have been attributed to chromosomal effects [2], hormonal effects [46] and social influences [32]. Regardless of the reason, cognitive differences between men and women are detected in the vast majority of studies. Cases of DSD again provide a unique platform to assess the possible contributing factors responsible for generating sex differences, as studies in individuals with CAH and CAIS have established unique hormonal and chromosomal influences. Spatial awareness and mental rotation ability reveal the strongest cognitive difference between men and women—with males out performing females [47, 48].

Spatial task assessments in cases of DSD are predominantly focused on individuals with congenital adrenal hyperplasia and to a more limited extent, CAIS. 46, XX females with CAH, on average out-perform their unaffected matched controls on mental rotation tasks and spatial relations tests [49-51]. Studies of this nature have also found that girls with CAH versus their

matched controls exhibit no enhancement in spatial ability, adding to the uncertainty of androgen influence on increasing spatial awareness on an XX background (Hines et al. 2003b). Some of these inconsistencies in the data may be attributed to variations seen between sub-types of CAH as opposed to an inaccurate determination of enhancements in spatial ability. It was found that spatial task enhancements show the strongest effect in girls with the severe salt wasting form of CAH, whereas those individuals with the simple virilizing form of the condition did not show a statistically significant difference (Hampson and Rovet 2015). In comparison, 46, XY males with CAH, appear to be de-masculinized on these features and perform worse than their matched controls on both measures [51, 52]. These findings are quite interesting as they allude to the possibility that high *in utero* androgen exposure generates different cognitive outcomes depending on sex chromosome complement. Males with CAH *a priori* would be expected to perform at least equally as opposed to underperform control males if the effect was exclusively due to excess testosterone. There may be both an optimal time and level of testosterone exposure during development that enhances such abilities, and if these levels too high *in utero* it may generate these observed outcomes in males with CAH.

Recently, individuals with CAIS were assessed using both traditional rotation tests and fMRI studies to determine how their brains responded to rotation tasks as compared to non-CAIS males and females. It was found that 46, XY CAIS women were slower to figure out the correct response in rotation test assessments as compared to their 46, XY male counterparts [53]. When focusing on neuroimaging, CAIS individuals were more similar to control women, showing less inferior parietal lobe activations during rotation tasks than they were to 46,XY males [53]. This study is quite remarkable as it demonstrates a strong role of androgenic influences on establishing mental rotation abilities on an XY background, despite the fact that males with CAH

(elevated T levels) perform worse than controls. Again, these findings suggest that timing and dose of testosterone during development may be critical to establish or enhance specific cognitive traits in humans. CAH individuals have more confounding long-term health factors, whereas in CAIS these additional variables are minimized. Thus, this leaves the testosterone responses in the brain as the most reasonable explanation for the observed spatial performance differences. To the best of our knowledge, no spatial performance examinations have been conducted on individuals with 5-α-reductase deficiency.

Another cognitive task where mild sex differences arise is the ability to target objects using hand-eye coordination, another male-typical feature [54]. In studies focusing on this trait, it was found that 46, XX CAH females were able to target both darts and ball throwing more accurately than their control counterparts [52, 55]. In males with CAH this trait seems to be unaffected, as both controls and affected individuals were statistically insignificant from one another in targeting abilities [52, 55]. It appears again that an *in utero* androgenic influence on an XX genetic background enables enhanced targeting coordination during adolescences and adulthood. This feature, unlike spatial ability, doesn't appear to be sensitive to the dose or timing of testosterone during development, as CAH males did not underperform their controls on these measures. This is another trait that has yet to be examined in individuals with CAIS and 5-α-reductase deficiency. Such work would greatly improve our understanding of androgenic and sex chromosome influence on shaping specific cognitive sex differences within the human brain.

Additional cognitive traits which have been assessed and show sex differences include male-dominate tasks involving spatial perception, maze navigation, mathematics, and various mechanical skills, and female-dominate tasks involving working memory and verbal fluency [56-58]. Many of these traits have been assessed in XX and XY CAH cases; however, the

cognitive differences that show the strongest sex and androgen influence are the highlighted spatial and targeting abilities. The works conducted in cases of DSD have helped expand our understanding of the influence of testosterone on both XX and XY genetic backgrounds with regards to altering spatial abilities and hand-eye coordination. These works have also demonstrated that the effect is most likely hormonally influenced, as CAIS individuals despite being XY cannot respond to androgen and therefore have spatial and cognitive traits in resemblance of XX females.

Brain structural variations in DSD cases: Unlike research focused on sexual behavior and cognitive differences, there exists surprisingly little work focused on the structural variations of the brain as a result of DSD. A small but growing body of literature has addressed this topic mostly in individuals with CAH. Early work established that both 46, XY males and 46, XX females with 21-hydroxylase deficiency exhibited significantly smaller amygdala volumes when compared to aged matched controls, a known sexually dimorphic brain structure [59, 60]. It was found that regardless of age, girls with CAH exhibited a bilateral reduction in amygdala volume, whereas CAH affected males in the study had a 20% unilateral reduction in only the left amygdala, which is also the same reduction percentage for the left amygdala in CAH females. Increases of amygdala volumes over human development have been observed in males which is attributed to arise from androgenic stimulation [61]. Interestingly, it was found in 46, XY boys with CAH, this increase with developmental age was only present in the right, seemingly unaffected amygdala; whereas XY controls in the study group followed previously established bilateral size increases [59]. The exact causes of these brain anomalies are still being addressed; however, there seems to be a clear sex difference in response, as males and females with similar

imbalances as a result of CAH show different patterns of amygdala reductions. A study focusing primarily on 46, XX non-CAH individuals taking long-term corticosteroid replacement therapies also identified a 20% reduction in the left amygdala volume and an 11% reduction in right hemisphere amygdala [62]. Collectively these findings indicate that the reduced amygdala volume is most likely due to an imbalance of glucocorticoids rather than excess androgens during development, as amygdala size is actually reduced in CAH patients despite high levels of *in utero* testosterone, whereas healthy male controls show increases in volume of the amygdala via androgen stimulation. These findings however do not offer a direct biological mechanistic explanation for observations of reductions in brain regions, but supports that imbalances in glucocorticoids and other steroids are the likely causative agents.

White-matter abnormalities: Another brain structural metric that has been assessed in XX and XY individuals with CAH are abnormalities in white matter distributions. MRI studies of adult patients with salt wasting, simple virilizing and even non-classical congenital adrenal hyperplasia have identified white matter hyperintensities in various brain regions [63-67]. Areas of the brain that seem to be most frequently affected in CAH patients are the periventricular regions, areas of the cerebellum and the corpus callosum. Incidences of temporal lobe atrophy have also been documented from MRI studies of CAH patients [65, 66]. The vast majority of cases assessed showing abnormalities in white matter across brain regions scored within the normal ranges of neurological function, indicating that despite these differences, the effect does not seem to impair proper brain functioning. These studies have all identified sub-clinical white matter variations, however the etiology and results of such findings remains unclear. It has been hypothesized that the logical causes of these outcomes results from hormonal imbalances during development,

long-term glucocorticoid replacement therapies after birth, and even due to the genetic mutation itself. It has also been proposed that the imbalance of hormones during development contributes to improper oligodendrocyte differentiation and in improper myelination [68]. In addition to adult studies, there have also been several documented cases of newborns with CAH exhibiting white matter anomalies [63, 64]. While it does appears that replacement therapies over a long-time frame may affect white matter, findings in newborns with CAH raise the notion that long-term glucocorticoid replacement therapies may not be the sole explanation for the disturbances in white matter. Investigating these unusual findings in more detail will enable a better understanding of the role that glucocorticoids or lack thereof have on shaping the structure of the developing male and female brain. Currently however, the exact cause and mechanism behind these results remain speculative.

Conclusions: Research focused on cases of DSD have helped the scientific community better understand the interplay between gonadal hormones and sex chromosome complement in regards to generating some of the sex differences observed in humans. These works have shed light on the likelihood that testosterone exposure as opposed to sex chromosomes is a larger contributing factor for guiding ones sexual orientation, and to a lesser extent gender identity. We see that 46, XX CAH individuals that have been exposed to *in utero* testosterone experience a greater degree of dissatisfaction in gender assignment in addition to above average levels of homosexual and bisexual fantasies, a proxy for sexual preference. As previously mentioned, other variables are present in CAH cases such as life-long medical interventions and psychosocial confounds. These variables may constitute an environmental factor that when coupled with biological pre-dispositions, generate variations in sexual orientation and gender identity. To say that sexual

orientation is determined solely by *in utero* hormonal milieu is unlikely. We see that the vast majority of CAH women despite having been exposed to above average levels of testosterone still identify as heterosexual as measured by both partners and sexual fantasies. The science of sexual orientation is still weakly understood at the mechanistic level, however, considerable amounts of research have formulated many possibilities as to the causes of same-sex attraction [69, 70].

The strongest evidence which adds support for the influence of testosterone in structuring gender identity comes from the work focused on 46, XY CAIS, where nearly all individuals researched indicate feelings typical of female gender. In addition to self-reports and clinical evaluations, recent fMRI studies have also demonstrated that CAIS women not only feel female, but also neurologically respond more similar to 46, XX women than 46, XY men when observing sexual images. However, new studies are continually emerging that suggest gender-identity and sexual orientation in individuals with CAIS is not as clear as once thought, and the rates of non-heterosexual and gender dysphoria may be much higher than currently stated. In addition to CAH and CAIS, 5-α-reductase deficiencies have also demonstrated the strong role of testosterone's ability to hormonally organize the human brain and influence adult gender identity and behavior. If early *in utero* exposure had no influence in guiding brain gender, it would be expected that there would be considerable difficulty with the female to male transition observed in pubertal years in those with 5-α-reductase deficiency. What we observe however, is an overwhelming majority of individuals with this condition comfortably transitioned into the new gender role at puberty, a world-wide observation occurring throughout many different types of social environments. Despite the convincing findings for the role of testosterone in generating

these observations, the influence of social and other environmental variables are also factors that require consideration.

Cognitive conclusions: Studying cases of DSDs has also provided insight into some of the biological parameters that generate sex differences in cognitive abilities such as visuospatial awareness and targeting ability. From studies using 46, XX CAH individuals it has been well established that *in utero* androgen exposures seems to enhance the ability to mentally rotate objects as well as improving hand-eye coordination during targeting tasks. This trait appears to be dependent on sex chromosome complement in addition to hormone exposure as 46, XY males with CAH actually perform worse than their matched controls, which is unexpected giving the fact that CAH males would have equal or elevated levels of circulating testosterone. This raises the notion as earlier mentioned that proper timing and dosage is also likely important for enhancing such abilities and simply having above average levels of testosterone during development would not generate a "super-male". CAIS provides another insight into this matter demonstrating that the ability to respond to testosterone on an XY background is critical to establish baseline spatial performance abilities. fMRI studies demonstrate that 46, XY CAIS had less inferior parietal lobe neuro-activation when performing spatial rotation tasks, a feature that resembles 46, XX females more than control genetic males. These fMRI studies on CAIS individuals once again minimizes social influences, and allows for a more unbiased assessment of the requirement of testosterone over genetic composition for shaping these cognitive performance sex differences.

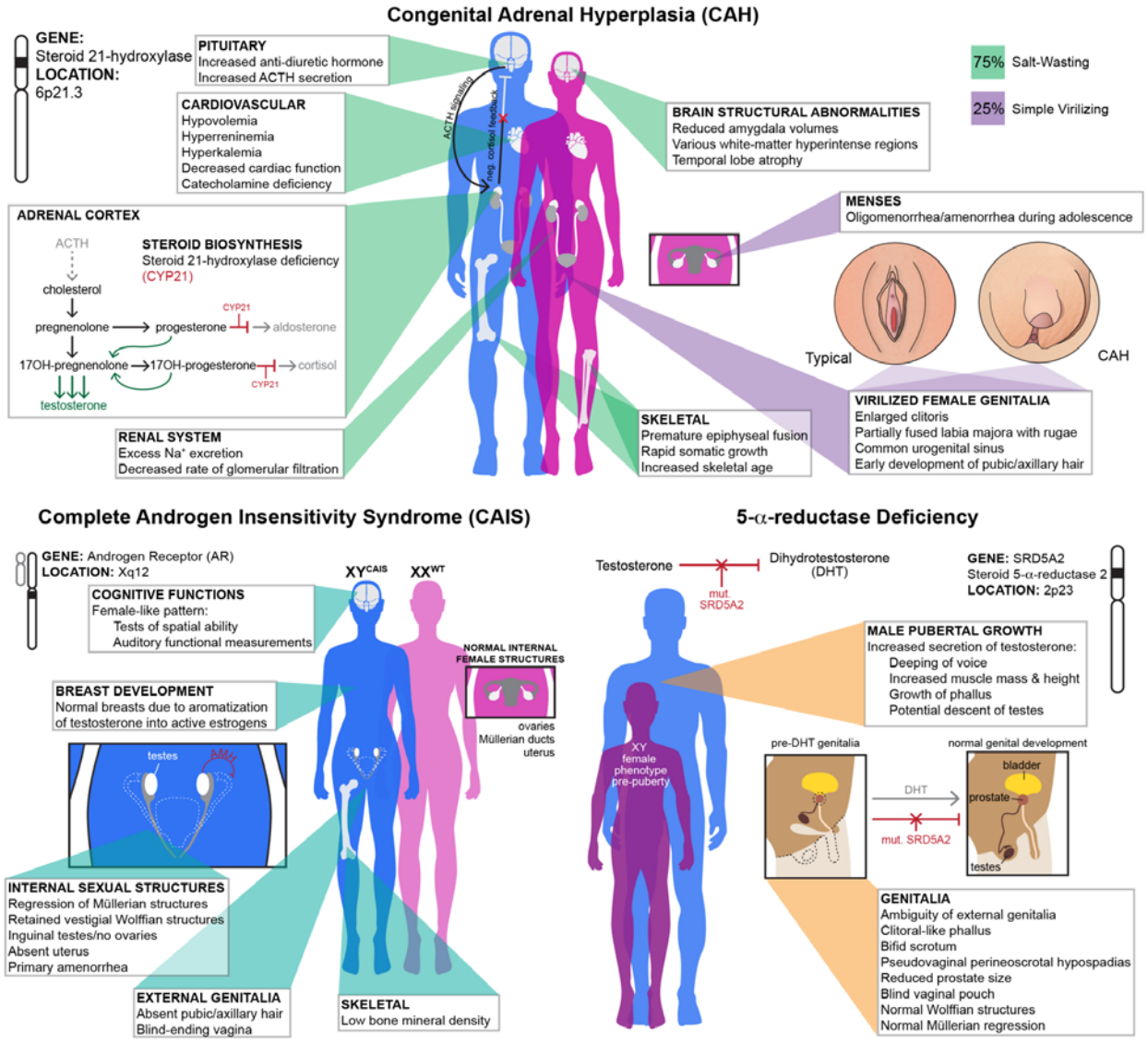
Structural Conclusions: From the MRI studies that have been conducted in patients with CAH it is clear that DSDs affect more than gonadal development. As highlighted, the central nervous system is highly sensitive to various hormones and imbalances of such can greatly affect

downstream behavior as well as overall brain structure. Variations in amygdala volume seem to be present in some individuals with CAH; however the effect is different depending on sex chromosome composition. Specifically, 46, XY males with CAH show unilateral reductions in the left amygdala, whereas 46, XX females with CAH show bilateral reductions in overall volume. Alterations on amygdala volume seem to be consistent with long-term glucocorticoid replacement therapies, as findings in non-CAH patients on such hormone regiments also show amygdala abnormalities. The documentations of white matter irregularities seem to be unaffected by chromosomal sex, and cause similar variations in both males and females with CAH. The explanations of these results are not in consensus and more research will be needed before causations can be associated with the unusual white matter findings. While limited, these discoveries have opened a new area of potential investigation focusing on the role of glucocorticoid influences on the developing brain in addition to the more frequently studied gonadal hormonal contributions. Unfortunately, no extensive structural studies have been conducted in patients with CAIS or 5-α-reductase deficiencies. These findings would be invaluable in determining the direct effect of testosterone on the structures that have been found to be altered in some cases of CAH by MRI studies. Future work focusing on outcomes in individuals with DSD will continue to aid in deciphering the contributions of chromosomal sex and hormones on shaping the sexually dimorphic human brain.

Conflicts of Interest: The authors of this study declare no conflict of interest that would have any bearing on the topics discussed in this review. MSB was the primary contributor to this article, conducting the literature review and manuscript preparation. AL prepared the graphic

designs and both AL and NV assisted in the literature review and manuscript preparation. EV guided the topics of discussion and oversaw the finalization of this review.

Figure 1-1: Medical overview of CAH, CAIS, and 5a-reductase deficiency



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Chapter 2

Sex-Differences and Gonadal Hormone Response in Cultured Mammalian Neural Stem Cells: A Contributing Element to Hormonal Brain Organization and Neurodevelopment?

Abstract: While numerous *in vivo* experiments have sought to explore the effects of sex and gonadal hormones on cellular proliferation and differentiation within the mammalian brain, far fewer studies, as reviewed here, have explored these factors using a direct *in vitro* approach. Generally speaking, *in vivo* studies provide the gold standard to demonstrate ones point, in the case of NSC biology however, there remain many unknown factors that likely contribute to observations made within the developed brain. For these reasons, using a NSC *in vitro* model may provide a better system to explore the direct effects of sex and gonadal hormone response; limiting the vast array of other influences on NSCs occurring during development and within adult cellular niches. These specific cellular models may have the ability to greatly improve the mechanistic understanding of changes occurring within the developmental brain during the hormonal organization process, in addition to other neuro-psychiatric sex-biased diseases.

Introduction: Investigating the phenomenon of hormonal organization of the brain has been a focal point within the field of neuroendocrinology since the seminal findings of Phoenix et.al was first published in 1959 [1]. Although, these findings elegantly demonstrated that exposing female fetuses to androgenic compounds resulted in altered adult sexual behavior, the exact mechanism behind this organization process remains to be fully elucidated. Significant works have built on the hormonal organization theory, and as such, have identified numerous sex-differences in addition to behavior, that are set in motion by gonadal hormone exposures *in utero* and during the perinatal period [2]. In addition to hormone exposure on the developing brain, it also appears that genetic composition [3, 4] [5, 6] and epigenetic modifications [7-9] significantly contribute to developing adult sexual behavior and sexually dimorphic brain structures within rodents and humans [10].

While the vast majority of the aforementioned studies have drawn conclusions based on analyses of gross brain tissue, other studies have looked at the direct effects of chromosomal composition and gonadal hormonal influence on specific cells comprising the central nervous system (CNS). Studies have highlighted the effects of testosterone and estrogens on various types of neurons and astrocytes, [11-15] however, few studies to date have explored these effects and the epigenetic consequences of such, on cultured neural stem cells isolated from the embryonic and adult brains.

Neural stem cells (NSCs) by definition are multipotent populations capable of giving rise to all of the main cell types that comprise the CNS, in addition to having self-renewal capacity [16]—the hallmark of any "stem" cell. There are two general groupings of neural stem cells, those present during early development which will be referred to as embryonic neural stem cells (eNSCs) and those that are maintained during/throughout adulthood (aNSCs). Embryonic neural stem cells are abundant, rapidly dividing and differentiating during early development, providing sufficient cellular numbers for proper brain formation. Adult NSCs however, are restricted to specific regions within the matured brain and remain under complex regulatory control within their respective niches [17-19]. Areas rich in quiescent NSCs during adulthood include the sub-ventricular zone (SVZ) and the sub-granular zone (SGZ) of the dentate gyrus (DG)[20]. Both types of NSCs retain stem properties; however, they appear to have different cellular features and protein expression patterns [16, 20]. This raises the notion that there are intrinsic and extrinsic distinctions to be made between adult NSCs and those present during early brain development, which will be particularly relevant to this review.

Research focusing on neural stem cells and adult neurogenesis has seen an explosion in the past two decades, which has been thoroughly described by Gage and Temple [21]. As noted,

despite intense investigation, few studies have sought to explore inherent sex differences and the role that gonadal hormones have in shaping neural stem cell biology, although studies indicate that such hormones influence adult neurogenesis within the DG, as reviewed elsewhere [22, 23]. The intent of this review is to highlight the *in vitro* work that has investigated these aspects in NSCs, exposing a novel role of gonadal hormone influence during early brain development and throughout adulthood.

Basal Sex-Differences in Cultured NSCs: During analysis of neural stem cells, there have been several studies that have identified inherent basal sex differences between XX and XY NSCs, independent of active androgen or estrogen exposure. One such sex-difference that has been found by several groups is the protein expression level of aromatase, an enzyme responsible for the conversion of testosterone into estradiol, which plays a vital role in hormonal organization of mammalian brains [24, 25]. Using NSCs isolated from the SVZ of adult 8-10 week old CYP19A1-EGFP BAC-mouse strain[26] and NSCs from the SVZ of 3 month old Long Evans rats[27], researchers were able to quantify aromatase expression using both a GFP reporter and total protein analysis. Those two independent groups found that aromatase expression, in the absence of gonadal hormones, displayed a significant male bias with regard to protein expression in adult NSCs. However, in a recent study conducted by our group utilizing RNA-sequencing, we did not identify any expressed aromatase transcripts in either male or female eNSCs[28]. While this discrepancy would typically seem contradictory, our group used NSCs isolated from the telencephalons of E-13.5 B57/B6/J mice, whereas the other two groups used adult isolated NSCs from both the rat and mouse SVZ. This difference in aromatase expression between adult and embryonic NSCs raises an interesting possibility that the effects of androgenic hormone

exposure on this cell type may have markedly different consequences depending on developmental stage. If aromatase is not expressed in murine embryonic NSCs, then during the *in utero* testosterone surge the effects on these cells are likely due to direct testosterone signaling. On the other hand, since adult mouse and rat NSCs do express aromatase, the downstream effects of pubertal androgenic exposure on these cells could be modulated either by direct testosterone action on the androgen receptor (AR)[29] or estrogen signaling, through its various receptors (ERα, ERβ, GPR30)[30].

As earlier mentioned, our group conducted a global transcriptional analysis using RNA-sequencing on eNSCs to determine if there were inherent sex-differences with regard to gene expression. To our surprise, we identified 103 transcripts that were differentially expressed between XX and XY eNSCs (FDR=.10) prior to gonadal hormonal exposure[28]. The vast majority of these transcriptional sex-differences were enriched in pathways predominately involved in cellular replication, possibly indicating that XX and XY eNSCs differently regulate proliferative states. While many of the identified differential transcripts were novel, this was the first study utilizing RNA-seq to probe for sex-differences of global gene expression in eNSCs. The Identified basal sex differences within eNSCs are quite interesting and provides a unique gene set for further exploration, it should also be evident that these findings represent a brief snap shot of developmental time. The developing body is highly dynamic and rapidly changing, as are neural stem cells [31]. When assessing early sex differences, it is vital to keep in mind that such findings have spatial and temporal fluctuations and are unlikely to remain static throughout the life of the animal.

Additional research focusing on improving NSC transplantation therapy for various neurological conditions, which still has significant hurdles to overcome [32], identified that both

age and sex of NSCs are contributing factors that should be taking into consideration[33]. Using adult NSCs isolated from the SVZ of 3 month and 20 month old Long-Evans rats, it was found that upon differentiation in retinoic acid, XY and XX NSCs displayed altered neurogenic and gliogenic potentials, which were both sex and age dependent. When measuring protein levels, it was determined that neuronal markers such as MAP2 (microtubule-associated protein2), GAP43 (GAP43 growth associated protein 43) and DCX (doublecortin) along with the oligodendrocyte marker CNPase, displayed male biased sexual dimorphic expression in differentiated NSCs isolated from 3 month old rats[33]. The same study found a reverse trend when looking at an astrocyte marker, GFAP (Glial Fibrillary acidic protein), which showed a very significant XX biased difference in expression levels. The early neuronal maker βIII Tubulin did not show sex differences in this study, however in a later study using immunofluorescence, it did appear that βIII Tubulin may be dimorphic in expression, post-differentiation[27]. It was identified that the expression of βIII Tubulin, along with those proteins that were sexually dimorphic (GFAP, MAP2, GAP43) were age dependent, and were significantly reduced in differentiated NSCs isolated from 20 months old rats as compared to those isolated at 3 months of age[33]. The authors conclude that XY NSCs may have inherent potential to differentiate into neuronal linages, while XX NSCs preferentially differentiate down a glia pathway when stimulated with 1μM retinoic acid, with age being a large contributing factor. It should be noted however, that while their differentiation process contained no exogenous gonadal hormones, the XY NSCs from both the 3 month and 20 month old rats were still exposed to endogenous testosterone prior to isolation. This endogenous exposure may have altered epigenetic programming and the conclusions found may not be due to inherent differences based on sex and age per se, but rather to this possible programming of NSCs induced by *in vivo* T exposure. This would seem like a

possible explanation, as the same group of authors attributed the findings of variances in differentiation potentials that had been uncovered earlier, could possibly be attributed to sexually dimorphic CYP19 (p-450 Aromatase) expression, which was uncovered in a their later finding[27].

In Vitro Effects of Estrogenic Compounds on NSCs

Estrogen Receptor(s) Expression: In order to determine the effects exerted by estrogens, predominantly 17β-estradiol (E2), on NSCs, it first had to be established that these cell types expressed the appropriate receptors. In 2002 one of the first studies assessing the role of E2 on NSC physiology *in vitro,* determined that both embryonic NSCs isolated from E15-E20 Wistar rats and NSCs isolated from the lateral ventricles of the same adult species, expressed both ERα and ERβ[34]. This group successfully demonstrated by western, that ERα was most expressed in eNSCs isolated at E15 and E17, and such expression was greatly reduced at E20 and within aNSC populations. The reverse was true for ERβ, which showed increased expression over the E15-E20 period as well as into adulthood [34]. In studies using aNSCs isolated from the SVZ of 3 month and 20 month male and female Long Evans rats, ERα and ERβ were again found to be expressed in both age groups, with expression levels being significantly higher in aNSCs isolated at 20 months of age. The same group of authors also claim that ERα and ERβ exhibit sexual dimorphic expression patterns in aNSCs at 3monts of age, but not at the 20 month stage[33]. In a more recent study published in 2016, the expression of estrogen receptors was aging confirmed in eNSCs isolated from E14.5-E16.5 Sprague-Dawley rat embryos. This study however also probed for a more recently identified membrane bound ER receptor, GPR30, and found that in addition to ERα and β, GPR30 was also strongly expressed, as measured by western[35]. While

receptors for estrogens appear to be present in both embryonic and adult NSCs isolated from the rat, the same does not appear to be true for NSCs isolated from embryonic mouse brain tissue. In 2013 a group assessed nuclear receptor expression within NSCs isolated from E-13.5 murine embryos using RT-PCR analysis and concluded that both ERα/β were not expressed or transcriptionally undetectable[36]. Similar findings demonstrating low/no detection of estrogen receptors was also corroborated by our groups complete transcriptomic analysis of E.13.5 mouse NSCs using RNA-Seq[28]. While these findings demonstrate that estrogen receptors are at least present in rat NSCs, they also highlight the notion that all NSCs should not be grouped together and the possible response to gonadal hormones such as E2 may be species specific and variable over different developmental time points.

Cellular Proliferation effects of estrogens on NSCs: Studies focusing on the effects of estrogens have predominately investigated how E2 affects cellular proliferation, as well as differentiation potentials within NSC populations. In the early 2002 study by Brännvall et.al, which first described the presence of estrogen receptors within these cell types, also assessed the physiological effects of estrogen exposure. Using eNSCs and aNSCs from Wistar rats, this group determined that when 10nM of 17β-estradiol was introduced to their culture media in the absence of the mitogen EGF (epidermal growth factor), there was a significant (7%) proliferation increase of eNSCs, as measured by BrdU positive cells [34]. This effect was not seen in their aNSC population, or when the ER antagonist ICI-182,780 was present. When the same assay was conducted in the presence of EGF, there was actually decreased proliferation of both eNSCs and aNSCs. This decrease in proliferation in the presence of EGF was attributed to upregulation of the Cyclin-dependent kinase (CDK) P21, as it was shown that protein expression of this cell-

cycle regulator was significantly increased in the presence of E2. The authors note that while E2 can moderately increase proliferation of embryonic derived NSCs; this effect is greatly dependent on other growth factors, as in their case, EGF. While a 2008 study investigating the effects of E2 on rat eNSCs was unable to replicate these initial findings; using a modified media composition, still demonstrated that estradiol alone was capable of increasing proliferation of eNSCs isolated from the telencephalons of E15 Wistar rat embryos[37]. The same conclusion was also drawn from both a 2010[38] and 2016 study, however, it was also determined that increased doses of E2 (50nM) actually had the opposite effect, resulting in a reduced proliferative state[35].

From these independent studies, it can be concluded that while E2 can significantly increase proliferation of rat eNSCs through ER action; these outcomes are greatly dependent on both the dose of estrogen and the site of NSC isolation. To our knowledge, only one study has assessed the effects of estradiol exposure on adult derived mouse NSCs and also found that E2 significantly increased cellular proliferation, using a Ki67 proliferation marker, regardless of chromosomal sex [26]. This study did not show estrogen receptor expression per se and as previously stated such transcripts have not been identified in murine eNSCs. However, given their results, it can be inferred that perhaps estrogen receptor expression, while not present at the embryonic stages, becomes expressed by aNSCs in the SVZ and stimulation with E2 elicits proliferative effects in the adult mouse also.

The effects of estrogens on NSC differentiation: In addition to proliferation, many of the studies assessing those effects have also investigated the role that E2 plays during the differentiation process. When eNSCs isolated from the rat were treated with 10nM of E2 while undergoing

differentiation, it was found that after 4 days, there was a significant increase in the ratio of βIII tubulin positive neurons over GFAP expressing cells. The same outcome was not seen when aNSCs were treated with E2 during differentiation, indicating again that embryonic and adult cells may have completely different responses to estrogens. This pro-neuron effect was attenuated when the ER antagonist ICI-182,780 was added to the media, indicating that these observations during differentiation were modulated in part by ERα/β signaling [34]. Recent publications came to similar conclusions, and determined that 10nM of E2 stimulated differentiation to proceed down a neural lineage as determined by the ratio of Tuj-1 (βIII Tubulin marker) to GFAP signal. This study also demonstrated again, that 10nM of E2 seems to be most optimal dose for stimulating this differentiation outcome, as 1, 20 and 50nM concentrations of E2 did not result in altered neuron/glia ratios[35]. There also appears to be no sex differences in the effects of E2 on NSC differentiation, at least in cultured adult murine cells, where both XX and XY lines showed increased neuronal staining when allowed to differentiate in the presence of 10nM of E2[26].

Two studies conducted by Okada et.al using embryonic derived rat NSCs were unable to replicate the findings showing preferential neuron differentiation upon stimulation with E2 [37, 38]. While this group did not observe increased neuronal potential, they did however determine that E2 increased differentiation of both CNPase positive oligodendrocytes and NG-2 positive oligodendrocyte precursor cells. The authors concluded (but did not demonstrate) that this effect was not a result of classical ER signaling, but rather membrane ER signaling, because when NSCs were pre-treated with ICI-182,780 the ratios of oligodendrocytes and precursor cells were still increased during differentiation in the presence of E2[37, 38]. It should also be noted, that while all studies are not in agreement with regard to pro-neuronal outcomes when NSCs are

stimulated with E2, there were also technical differences between experiments that may have played a factor. The studies that identified E2 as a pro-neuronal stimulant [34, 35] grew their NSCs in the presence of EGF, whereas the studies that were unable to replicate these findings and saw pro-oligodendrocyte differentiation[37, 38] grew their NSCs with the mitogen FGF-2(Fibroblast growth factor 2). It has been demonstrated that murine embryonic NSCs express FGF and EGF receptors in a temporal and spatial manner, and as such, respond to these mitogens in different ways[39]; highlighting the importance of what otherwise may seem like a minor technical difference between studies.

Conclusions of Estrogen Effects on NSCs: Prior to these *in vitro* applications using NSCs, groups have demonstrated the effects of sex and estrogens on eliciting both cellular proliferation and increased neurogenesis within the adult rodent brain; predominantly within the DG region of the hippocampus[23]. *In vivo* studies have demonstrated that cycling female adult SD rats display altered states of cellular proliferation within the DG, in accordance with various time points during the estrous cycle. Increases of cell proliferation were observed in the DG during proestrus, when circulating levels of estrogens were at their peak and subsequently declined during estrus, when estrogens were at lower levels [40]. However, if cellular proliferation is assessed prior to the onset of the estrous cycle, it appears that sex-differences exist; showing a male bias increase in cellular proliferation within the same brain region, as measured by BrdU labeling[41, 42]. These reviewed *in vitro* applications (**Table 1**) have also drawn similar conclusions, in at least cells isolated from embryonic stage SD rats, where E2 alone can elicit increased cellular proliferation. Interestingly, these proliferation increases were not seen in adult isolated NSCs from the SVZ, which may indicate that while estrogens elicit proliferation in the

DG, the same events may not occur within the SVZ. Additionally, the same strain of rat was not used for all studies, and data indicates that cellular proliferation within the DG is variable between strains [41], a factor that should be taken into consideration. *In vitro* evidence also supports that both dose of estrogen as well as other modulators such as EGF and FGF, contribute to the observed effects, as E2 in the presence of such mitogens actually causes a reduction in proliferation. Similar outcomes have also been observed in proliferating cells within the DG, where E2 dosage was a contributing factor, as well as the presence of other variables, such as progesterone, which essentially reverses the increased proliferation effects of estrogen[43].

In vitro studies (**Table 1**) have shown that NSCs isolated from the adult mouse SVZ show increased proliferation in the presence of E2, but *in vivo* approaches have observed the opposite; indicating that estrogens decrease proliferation within the SVZ[44]. When focusing on cellular propagation within the mouse DG, It was found neither sex nor estrogens stimulated adult neurogenesis or increased cellular proliferation in the C57/BL6 mouse strain [45]. This again highlights the notion that there are important differences to be made between mammalian species and site of study; grouping NSCs into a one size fits all approach is not recommended.

Based on several *in vitro* studies, it can be concluded that estrogen exposure on NSCs isolated from embryonic regions elicits altered differentiation potentials; increasing the ratios of neurons over GFAP expressing glia cell types. This effect appears to be restricted to embryonic stages, as estrogen stimulation on aNSCs *in vitro* did not have the same outcome, an effect also observed within the adult DG post-estrogen treatment[46]. However, as noted, not all groups observed this difference in neuron/glia outcome; those that did not detect a pro-neuronal outcome as a result of estrogenic stimulation, did detect an increase in oligodendrocyte differentiation *in vitro*.

Generally speaking, both the *in vivo* studies and *in vivo* studies have drawn similar conclusions when assessing the effects of estrogen stimulation on NSCs in culture, or progenitor cells within the adult rodent brain. Despite a growing body of evidence now indicating that estrogen stimulation can have dramatic epigenetic effects within the brain [47, 48], no study to date has directly assessed these effects on neural stem cells or progenitor cells within the brain. Seeing as these cells respond to gonadal estrogens, these *in vitro* models of NSCs will prove to be extremely useful for the study of epigenetic changes ensuing post-estrogen stimulation. These methods will limit the numerous variables occurring within the mammalian brain and allow for a more direct assessment of estrogen influence on epigenetic alterations such as DNA methylation, histone modifications and the ensuing gene expression outcomes.

In *Vitro* Effects of Androgenic Compounds on NSCs:

Androgen Receptor Expression: While several *in vitro* studies have provided evidence supporting the role of estrogens in proliferation and differentiation of cultured NSCs, few studies have addressed how such cells respond to androgenic compounds; despite known androgen response in other types of stem cells[49]. In 2005, Brännvall et.al demonstrated that both embryonic and adult rat NSCs derived from the SVZ of Wistar rats expressed the androgen receptor (AR). Using RT-PCR and western blots, the group showed that AR expression was most abundant in embryonic NSCs as compared to aNSCs, with peaks of expression occurring between gestational days E-15 to E17[50]. Androgen receptor also appears to be expressed in in both XX and XY aNSCs isolated from the adult mouse SVZ, and does not appear to show a sex-difference in protein expression. Unlike the results demonstrating low/no estrogen receptor gene expression in mouse eNSCs, this same study[36], along with our groups RNA-seq findings, did

identify that AR was transcriptionally detectable, and non-dimorphic in both male and female murine NSCs isolated from gestational day E13.5 embryos[28]. While limited, these independent studies identified that both embryonic and adult NSCs isolated from the rat and mouse express AR and therefore should responsive to various types' androgens.

Cellular Proliferation effects of androgens on NSCs: When both embryonic and rat aNSCs were cultured in the presence of the androgen Nandrolone (19-nortestosterone) along with the mitogen EGF, proliferation was decreased by 30% in eNSCs and 20% in aNSCs. However, if cultured in the absence of EGF with 10nM Nandrolone, there was roughly a 7% increase in proliferation of eNSCs, but this effect was not observed in adult female derived NSC populations. It was determined that these various alterations in proliferation were modulated in part by androgen receptor action, as treatment with the AR antagonist Flutamide quelled some, but not all of Nandrolone's effects [50]. Again, the authors probed for differences in various cell-cycle regulators that may be responsible for proliferation changes as a result of androgen exposure and determined that unlike their findings using E2[34]; CDK P-21 was not upregulated as a result. As concluded by the group, despite similar proliferation differences in response to E2 and Nandrolone in the presence of EGF, the pathways responsible for such outcomes appear to be modulated by different mechanisms of action.

When assessing the effects of various concentration of testosterone (T) on the proliferation of murine aNSCs, Ransome et.al demonstrated that while 1nM T did not produce proliferative differences, 10 and 50nM of T significantly increased proliferation of XX and XY aNSCs, even in the presence of both EGF and FGF-2. The increased proliferation as a result of T exposure was determined to result from Erk phosphorylation induced by MEK-1, as sex hormone

influences were ablated in the presence of U0126, a MEK1 phosphorylation inhibitor[26]. This particular study also found interesting sex-differences in how testosterone elicited these responses in aNSCs. It was determined that while T induced proliferation in both sexes, XX aNSC growth was inhibited by Flutamide, but XY aNSCs were still capable of increased proliferation even when AR was actively subdued[26]. This demonstrates that the conversation of T into E2 via aromatase can also modulate cellular proliferation in XY but not XX cells, highlighting the fact that male and female adult NSCs have differential response mechanisms when exposed to gonadal hormones. These two studies indicate that androgenic compounds can have significant effects on NSC proliferation, albeit in opposite directions, perhaps again revealing that different species of mammal NSCs respond differently to gonadal hormone stimulation.

The effects of androgens on NSC differentiation: Both of the groups that have assessed proliferative effects of testosterone on NSCs also addressed the role of androgens on differential outcomes post NSC differentiation. It was determined that if Nandrolone was present during a 5 day differentiation process of rat eNSCs, the proportion of βIII tubulin expressing neurons was higher than GFAP expressing cells, an effect not seen in adult NSCs. This effect was ablated when the 3μM of Flutamide was present, indicating that this effect was modulated by androgens binding AR [50]. The 2015 Ransom et.al study, also determined that the presence of 10nM T during a 2 day murine aNSC differentiation, resulted in an increase of βIII tubulin positive cells, in both XX and XY lines[26]. Like E2, it appears that androgens also have a pro-neuronal influence during differentiation of embryonic NSCs from the rat and adult NSCs from the

mouse. Due to lack of experimental evidence, it remains unclear if the same differentiation outcome occurs in embryonically derived murine NSCs.

Transcriptional and Epigenetic Effects of Androgen Exposure on NSCs: To the best of our knowledge, the findings published by our group in 2016, is the only study assessing the global transcriptional and epigenetic changes that occur as a result of testosterone propionate (TP) exposure on NSCs. Using murine eNSCs, we determined that 20nM of TP resulted in 2854 transcriptional differences on a XX background, and 792 gene transcript expression differences in XY eNSCs, using a false discovery rate of 10% (FDR=0.10)[28]. While TP had more robust effects on a XX genetic background; 600 of these differentially expressed transcripts were mutually shared between the sexes, indicating that there are both sex-chromosome independent as well as dependent effects of TP exposure on eNSCs gene transcription. In addition to detecting gene expression differences as a result of androgen exposure, we determined that TP significantly reduced global levels of 5-methylcytosine during active exposure, an epigenetic modification that was apparently transmissible to daughter cells in the absence of androgen. Another epigenetic modification that showed androgen sensitivity was acetylation levels of histone tails, which were found to be modified in a sex-dependent manner[28]. While additional studies will be necessary to solidify these findings, the data presented indicates that androgens not only have the capacity to greatly influence gene transcription, but also various epigenetic modifications within eNSCs.

Conclusions: Unlike studies involving estrogen stimulation on NSCs, very few studies have demonstrated the effects of androgens on the same cells. The limited evidence supports that

androgens can induce proliferation of embryonic rat NSCs in the absence of growth mitogens, however if present in culture, androgen stimulation reduces proliferation. This effect was not observed in adult isolated NSCs from the rat SVZ, indicating that androgen stimulation on embryonic populations are markedly different than when assayed using adult NSCs (**Table 1**). *In vivo* studies have also found that that androgenic stimulation does not increase proliferation in the DG of adult rats, nor does there appear to be AR expression within that region[51, 52]. These findings indicate that AR is expressed and responsive to androgens within adult cells isolated from the SVZ, however not from other neurogenic niches such as the DG. The same also appears to be true for SVZ isolated NSCs from the mouse, where testosterone exposure can also increase proliferation in both XX and XY NSCs.

Differentiation outcomes appear similar to those found with estrogen stimulation, showing that androgenic treatment can increase the ratio of neurons to glia during cultured differentiation of embryonic rat NSCs and adult isolated murine NSCs from the SVZ (**Table 1**). Despite *in vivo* studies showing that androgen did not increase proliferation in the DG, it was demonstrated that prolonged androgen exposure increased neurogenesis by enhancing the survival of adult born neurons within this region[51]. Despite variable outcomes from *in vivo* studies, there is still very convincing evidence for the role of androgenic influence on adult neurogenesis [22]. Our groups findings indicating that testosterone exposure can significantly reduce DNA methylation and alter histone tail acetylation within murine eNSCs raise the notion that androgenic exposure has significant consequences on the NSC epigenome. While in vivo findings have shown that testosterone can alter DNA methylation[7] in gross brain regions, our group was the first to show that these effects are also observed in cells at early time points during brain development. This raises a possibility that early androgen exposure may elicit significant

developmental, as well as behavioral influences outcomes, by reprogramming the epigenome of NSCs. These outcomes may contribute significantly to normal male behavioral development, as XY rodents with inactive AR (Tfm model) display reduced male-typical aggressive and sexual behaviors in adulthood, despite functional estrogen receptors [53]. While still in the very early stages, it is likely that deeper investigations into the roles of estrogens and androgens on neural stem cells will significantly improve our knowledge of gonadal hormone response on the embryonic and adult brains. These future investigations will likely lead to a more complete and comprehensive understanding of the early mechanisms behind hormonal organization of the mammalian brain.

Table 2-1: Overview of all studies investigating the effects of both androgen and estrogenic response in adult and embryonic neural stem cells

				Receptor Expression			Estrogenic Effects on NSCs					
							Differentiation Outcomes					
Year	Species	NSC Type	NSC Age	ER α Expression	ER β Expression	GPR30 Expression	Pro-Neuron	Pro-Glia	Pro-Oligodendrocytes	Proliferation Changes	Epigenetic Effects	Authors
2002	Wistar Rat	Embryonic	E15-E20	+ (WB)/(RT-PCR)	+ (WB)/(RT-PCR)		+	–	–	↑(–EGF) /↓ (+EGF)		K. Brännvallet al.
2002	Wistar Rat	Adult (SVZ)		+ (WB)/(RT-PCR)	+ (WB)/(RT-PCR)		–	–	–	none (–EGF)/ ↓ (+EGF)		K. Brännvallet al.
2008	Wistar Rat	Embryonic	E15	+ (RT-PCR)	+ (RT-PCR)		–	–	+	↑(-FGF-2) /↓ (+FGF-2)		M. Okada et al
2010	Long-Evans Rat	Adult (SVZ)	3 Month	+ (WB)	+ (WB)							J.Waldron et al.
2010	Long-Evans Rat	Adult (SVZ)	20 Month	+ (WB)	+ (WB)							J.Waldron et al.
2010	Wistar Rat	Embryonic	E15	Shown in 2008	Shown in 2008		–	–	+	↑(-FGF-2)		M. Okada et al
2013	Mouse	Embryonic	E13.5	– (RT-PCR)	– (RT-PCR)							A. Androutsellis Theotokis et al.
2015	C57/BL6J Mouse	Adult (SVZ)	8-10 Weeks				+	–	–	↑(+FGF-2+EGF)		M.Ransom et al.
2016	Sprague-Dawley Rat	Embryonic	E14.5-E16.5	+ (WB)	+ (WB)	+ (WB)	+	–	–	↑(+EGF)		L. Zhang et al.
2016	C57/BL6J Mouse	Embryonic	E13.5	– (RNA-seq)	– (RNA-seq)	– (RNA-seq)						M.S. Bramble et al

Year	Species	NSC Type	NSC Age	AR Expression	Pro-Neuron	Pro-Glia	Pro-Oligodendrocytes	Proliferation Changes	Epigenetic Effects	Gene Expression	Authors
2005	Wistar Rat	Embryonic	E12–20	+ (WB)/(RT-PCR)	+	–	–	↑(–EGF) /↓ (+EGF)			K. Brännvallet al.
2005	Wistar Rat	Adult (SVZ)		+ (WB)/(RT-PCR)	–	–	–	none (–EGF)/ none (+EGF)			K. Brännvallet al.
2013	Mouse	Embryonic	E13.5	+ (RT-PCR)							A. Androutsellis Theotokis et al.
2015	C57/BL6J Mouse	Adult (SVZ)	8-10 Weeks	+ (WB)	+	–	–	↑(+FGF-2+EGF)			M.Ransom et al.
2016	C57/BL6J Mouse	Embryonic	E13.5	+ (RNA-Seq)					DNA Methylation Sex-Specific Changes Acetylation	Sex-Specific Changes	M.S. Bramble et al.
				Receptor Expression	Differentiation Outcomes			Androgenic Effects on NSCs			

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Chapter 3:

Sex-Specific Effects of Testosterone on the Sexually Dimorphic Transcriptome of Embryonic Neural Stem/Progenitor Cells

Abstract: The mechanisms by which sex differences in the mammalian brain arise are poorly understood, but are influenced by a combination of underlying genetic differences and gonadal hormone exposure. Using a mouse embryonic neural stem cell (eNSC) model to understand early events contributing to sexually dimorphic brain development, we identified novel interactions between chromosomal sex and hormonal exposure that are instrumental to early brain sex differences. RNA-sequencing identified 103 transcripts that were differentially expressed between XX and XY eNSCs at baseline (FDR=0.10). Treatment with testosterone-propionate (TP) reveals sex-specific gene expression changes, causing 2854 and 792 transcripts to become differentially expressed on XX and XY genetic backgrounds respectively. These findings indicate that testosterone exposure on XX cells may have a more robust effect with regards to altering gene expression than on XY cells. It was also found that by exposing XX eNSCs to TP 42% (43/103) of the original 103 basal sex differences that existed became masculinized and shifted towards a XY typical gene expression pattern. We also determined that 25% (26/103) of basal sex differences were actually feminized in an XY background post-TP. This suggests modular effects of TP exposure on sexual brain development. Additionally, within the TP responsive transcripts, there was enrichment for genes which function as epigenetic regulators that affect both histone modifications and DNA methylation patterning. This enrichment may suggest that in addition to gene transcriptional changes, the epigenome is also being actively modified in the presence of androgenic compounds. Collectively, these transcriptional findings highlight an unknown component of androgen action on cells within the developmental CNS and add to a novel proposal of action by which early hormonal organization is initiated and maintained.

Significance Statement: Prior investigations have addressed the role of sex chromosomes and sex hormones on specific brain regions; however the role of these factors on embryonic neural stem cell gene expression was unknown. Our data have revealed that XX and XY NSCs have inherent sex differences with regards to gene expression. We have also shown that testosterone has markedly different effects on gene regulation, depending on the sex chromosome complement of the cell. Most interestingly we have demonstrated that on a XX background, testosterone has the ability to masculinize portions of basal gene expression, as well as feminize basal expression on an XY background. These findings contribute towards a new model of how organization of the brain is initiated and permanently maintained.

Introduction: Pivotal studies on the rodent developing brain led to the organizational-activational hypothesis, which states that exposure to gonadal hormones are a strong contributing factor in the early development of the sexually dimorphic male and female brain [1]. The role of gonadal hormones on sexual differentiation, and brain masculinization has been investigated in the rodent model over the past century, and has been comprehensively reviewed elsewhere[2, 3]. Despite extensive investigation looking at the direct effects of chromosome complement [4-7], gonadal hormones and epigenetic influences [8-12] on the developing brain, there is a paucity of information that has addressed the multifaceted role of these factors on the progenitor cells that generate the central nervous system—neural stem cells (NSCs). To date, sex differences in NSCs have been limited to showing that sexual dimorphisms exist in the protein expression of P450 Aromatase (CYP19A1) in the adult rat and mouse NSCs isolated from the sub-ventricular zone (SVZ) [13, 14]. This enzyme is responsible for the conversion of testosterone derivatives into active estrogens and is associated with rodent brain masculinization[15]. Furthermore, expression of P450 Aromatase correlates with differences in cellular proliferation, and

differentiation[13, 14]. In addition to sexual dimorphisms, NSCs have also been shown to respond to gonadal hormones in a developmentally and/or site specific manner. For example, 19-Nortestosterone, and 17β Estradiol can negatively regulate the proliferation of NSCs derived from the lateral ventricles of adult rat brains, whereas 17β Estradiol exposure on embryonic derived rat NSCs seem to have a positive regulatory effect, as well as increasing neurogenesis [16, 17]. These studies have established that neural stem cells respond to gonadal hormones, albeit in different ways depending on either developmental time and/or site of isolation, however, the molecular and genetic changes that occur as a result of early hormone exposures on this important cell type generally remain elusive. We aim to deepen the understanding of the effect that gonadal hormones have on the early stem/progenitor cells of the developing central nervous system—and identify underlying mechanisms behind cellular programming and maintenance of adult sex differences in the mammalian brain.

Here we present a transcriptomic approach, utilizing RNA sequencing of embryonic mouse neural stem cells (eNSCs), revealing sexual dimorphisms in gene expression at a time point prior to the onset of endogenous gonadal hormone surges, namely testosterone. In addition, we demonstrate the strong sex-specific transcriptional effects of testosterone on eNSCs, which not only equalizes numerous basal sex differences on a XX background, but serves to de-feminize and masculinize gene expression. These findings are the first to uncover basal sex differences in eNSC gene expression, and further provide a dataset of sexually dimorphic testosterone-responsive genes. These activational changes that occur as a result of androgen exposure on NSCs may be the initiating steps of the process leading to the observed long-term effects of hormonal influence and shed light on the biological basis of certain sex-biased neuro-psychiatric disorders[18].

Results:

Generation of multipotent neural stem cells:

To determine the transcriptomic effects of testosterone exposure on XY and XX undifferentiated eNSCs, we harvested multipotent neural stem cells from embryonic 13.5-14 C57BL6/J mice and followed the experimental timeline outlined in (Fig. 3-1A). After a growth period of 5 days, we stained for an accepted NSC marker, Nestin, which showed strong expression in both XX and XY cell types (Fig. 3-1B) demonstrating an accepted NSC staining profile. As identified by RNAseq reads, our eNSCs are also Pax6 and Sox2 positive, further confirming accepted markers of eNSCs within our experimental cell type (Fig. 3-1C) [19, 20]. In addition to staining markers of eNSCs, these cells also give rise to both mature neurons and astrocytes, as demonstrated by the GFAP and Tuj-1 staining, (Fig. 3-1B) along with the dramatic increase in transcript expression of both astrocyte and neuronal markers, 21 days post-differentiation (Fig. 3-1D). Taken together, our immunofluorescence profile, gene expression data, and the differentiation potential, validate the multipotent characteristics of our experimental cell line.

Male and Female Embryonic Neural Stem Cells Show Sexual Dimorphisms in Gene Expression:

First, we wanted to determine if eNSCs exhibited any sexual dimorphisms with regards to gene expression, prior to androgenic hormone exposure. RNA sequencing analysis comparing XX and XY undifferentiated eNSCs found that there was a strong correlation between global gene expression (ρ=.986) and identified over 1000 genes that were greater than 2-fold differentially expressed between sexes (Fig 3-2A). After a FDR correction of 0.10, we identified 103 transcripts that were significantly differentially expressed (Fig. 3-2B) (Table 3-1). This effect is attributed to arise from an inherent sex chromosome effect, as these cells have not been

extensively exposed to gonadal hormones prior to isolation from embryos. Of the differentially expressed transcripts in NSCs, 74% showed a decreased expression, and 26% showed increased expression in an XX background relative to XY (Table. 3-1).

A priori, we expected certain genes to be differentially expressed between sexes, specifically those genes known to be expressed only in females and those present on the Y chromosome. As an internal control, we indeed found that Kdm5d and Xist were the two most differentially expressed genes between XX and XY eNSCs at baseline. Given their known sexually dimorphic expression patters [21] further supports the validity of our data set (Table 3-1). For additional confidence, we selected 12 of our differentially expressed genes to validate by a secondary approach of qPCR, which showed similar expression differences that were identified within our RNA-seq dataset (Fig. 3-3). Having uncovered sexual dimorphic gene expression in embryonic neural stem cells, we queried if these 103 genes were enriched in any known biological processes. Using DAVID bioinformatics software [22, 23] , we found that the vast majority of these differentially expressed genes were enriched in biological processes known to control cellular proliferation (Fig 3-2C). Interestingly, other pathways including those involved in cellular communication and neuronal differentiation were also enriched among sexually dimorphic genes (Fig. 3-2C).

Testosterone Alters Global Gene Expression in Both XX and XY eNSCs: After identifying sexually dimorphic basal gene expression differences, we next determined if testosterone had an effect on global gene expression. We treated XX and XY eNSCs with a single dose of TP to a final concentration of 20nM for a period of 5 days [24]; RNA-seq was performed and analyzed to determine the effects of gonadal hormones on the transcriptome (Fig. 3-1A). We found that in

both XX and XY eNSCs, testosterone had a large global effect on gene expression when compared to basal transcript levels (Fig. 3-4A/B). When we screened for genes that had a greater than a 2-fold $\log_2$ change in expression as a result of testosterone exposure, we detected over 4000 such events in both sexes. We next queried the data set to determine if the effect of testosterone was the same in both sexes at the global level of transcription. To determine this, we took the $\log_2$ value of gene expression for both sexes in the presence of testosterone and divided that by the baseline $\log_2$ expression, giving us a ratio based on both the sex chromosome and testosterone effect. Once these ratios were derived and plotted, we see the effect of testosterone was markedly different between sexes (Fig. 3-4C). We see that the majority of genes cluster in the 1:1 regions of the plot indicating that testosterone does not affect all genes, as expected. Most interestingly however, we found that there are a significant number of gene points that are widely dispersed away from the center cluster, indicating that the effect of testosterone on altering gene expression appears to be dimorphic at the global level, depending on the sex chromosomal composition of cells that are being exposed to testosterone (Fig. 3-4C).

Sex Chromosome Dependent Effects of Testosterone Exposure:

Once we established that testosterone had global effects on gene expression, we determined which genes were differentially expressed in each sex utilizing a FDR= .10. We found that 2854 genes were differentially expressed (FDR=0.10) in XX eNSCs post-testosterone exposure, compared to baseline expression values (Fig. 3-5A). Of the 2854 differentially expressed genes, half were up regulated and half were down-regulated after treatment (Supplemental Table. 3-2). We then determined if the effect of testosterone was the same on an XY background. We found that 792 genes were differentially expressed in male eNSCs as a result of TP exposure (Fig 3-

5B). On this genetic background we found that 70% of genes were downregulated, and 30% of the 792 differentially expressed genes were up regulated in the presence of testosterone (Supplemental Table. 3-3). Despite the fact that testosterone had a more robust effect on XX eNSCs than on XY, the two groups shared 616 genes that were mutually affected by TP, constituting 78% of the total genes affected in XY cells (Fig. 3-6A). Next, we determined if there were perhaps functional groups that were being affected within the differentially expressed genes postTP treatment that were unique to each sex. To further analyze possible functional groupings of sexually dimorphic patterns of gene expression we subjected the top 200 most down regulated genes from each genetic background to pathway analysis using the recently available Broad Institute's GeNets software. Each gene set was analyzed with the seven publically available algorithms (PPI, ConcensysPathDB, GeNets Metanetwork V1.0, Geo, Achillies, Blast and CLIME). Pathways identified were done so using three main criteria, 1) all associations had to be significant, 2) all associations had to have more than 2 genes in their functional groupings, 3) all associations had to be present in more than one algorithm queried. We identified two significant pathways that were unique to each sex, implicated by multiple algorithms in our post-TP exposed cells. Pathways involved in regulating potassium channels were the most downregulated in XX cells (Fig. 3-5C). However, in XY cells, TP downregulated a pathway involved in internalization of an epidermal growth factor receptor, ERRB1 (Fig. 3-5D). These findings demonstrate that the effect of TP exposure is distinctly different between the sexes of eNSCs, causing pathways unique to each sex to become potentially differentially regulated.

Sex Chromosome Independent Effects of Testosterone Exposure: Having found that testosterone affected transcription in a sex chromosome-specific manner, we investigated how the 616

mutually affected genes between XX and XY were responding to TP. After plotting the delta FPKM (log_2) expression values for both sexes pre and post-TP exposure, we found that the shared gene group responded quite similar to testosterone (Fig. 3-6A). The majority of the mutually affected genes responded in corresponding directions after being exposed, regardless of sex chromosome complement, as shown by the strong correlation coefficient of 0.96 (Fig. 3-6A). Although the majority of mutually affected genes responded in similar directions, a small but interesting group of genes responded either inversely, or with greater magnitude after hormone treatment depending on genetic background. The top ten most disproportionally affected genes that were shared between the sexes are *Atf5, Cth, Nupr1, Cox6a2, Trib3, Hsd17b1, Fgf21, Adm2, Atp2a3* and *Tceal1* (Fig.3-6A). Having identified enriched functional groups that were uniquely downregulated in each sex (Fig. 3-5 C/D), we next determined pathways which were up-regulated as a result of TP exposure irrespective of sex chromosome complement. We queried if the 200 most up regulated genes in the mutually affected set were enriched in any known biological functional categories [22, 23]. We found a strong enrichment in pathways representing nucleosome organization/assembly and general DNA architecture which were comprised of three communities of gene groups that were significantly upregulated in both XX and XY embryonic neural stem cells after hormone treatment (Fig 3-6B). These enriched pathways that are altered in eNCSs from exposures to androgenic hormones are important for both DNA compaction in addition to how the genetic code is regulated, transcribed and translated.

Testosterone has Both Masculinizing and Feminizing Effects in eNSCs: The effects of testosterone on the brain are often characterized in terms of how masculinized a trait becomes

over time, such as the case in the hormonal organization theory. We investigated if the basal sex differences that were previously identified (Table. 3-1), had become masculinized (more similar to XY transcript reads) or feminized (more similar to XX transcript reads) in gene expression after TP treatment. We found that 43 out of the 103 original sex differences post-TP exposure on a XX background were at least equal to the FPKM expression values for the same gene on a XY background prior to TP treatment, indicating full masculinization of such gene expression (Fig. 3-7) (Table. 3-1). Unexpectedly, we also identified 26 genes on a XY background that actually became completely feminized as a result of exposing such cells to TP (Fig. 3-7) (Table 3-1). These feminizing and masculinizing events as a result of exposing embryonic neural stem cells to a male sex hormone are perhaps identifying key genes responsible for establishing typical male gene expression within the cells of the developing central nervous system. In addition to identifying key genes involved in typical-male brain development, the findings also shed light on early transcriptional events that may be occurring in hyper-masculinized female development, as seen in cases of XX congenital adrenal hyperplasia (CAH). These findings demonstrate the ability of gonadal hormones to modulate effects of sex chromosome composition, resulting in diminished XX and XY basal gene expression differences, making such expression more similar in nature.

Long-term Effects of Testosterone Exposure on the Transcriptome of eNSCs:

After we identified that testosterone was able to alter gene expressing in both sexes of eNSCs we next determined if these gene expression differences were maintained in the daughter cells of the TP exposed eNSCs. We found that once the cells were passaged and allowed to grow for an additional 5 day period prior to RNA-sequencing in the absence of testosterone (Fig. 3-1A), no

differential gene expression was maintained using an FDR=.10. These data show that while testosterone does have a strong activational effect with regard to altering gene expression; these effects are not maintained within in the daughter cell population in the absence of testosterone, indicating that these transcriptional effects occur only while the hormone is present (data not shown).

Methods: *Animal Care and Neural Stem Cell isolation*: C57BL/6/J XX and XY mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed at the UCLA Animal Care Facility. Animals were maintained at 20°C with a 12-h light/12-h dark cycle, provided *ad libitum* with food and water. The study was approved by the University of California, Los Angeles (UCLA) Committee on Animal Research and was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. At eight weeks of age three dams and three males were mated separately to generate the biological triplicate basis, and the gestation was timed for 13.5 days. After the appropriate gestation time, the pregnant dams were humanely sacrificed, and immediately prepped for embryo extraction. Embryos were separated, followed by the removal of the brain which was then placed separately into dishes containing cold PBS/2% glucose. The ganglionic eminences from both hemispheres were removed and placed into 1.7ml Eppendorf tubes supplemented with cold PBS/2% glucose. This procedure was repeated for every embryo in each of the three dams following standard technical protocols [25]. Following isolation of the ganglionic eminence regions, the CNS tissue in each tube was triturated using a P-200 until no tissue clumps were visible prior to being passed through a 40μM cell strainer. After straining, all cells from each brain region were plated into 6-well culture dishes (Falcon) and suspended in

Complete Embryonic Neurocult™ Proliferation Media (STEMCELL Technologies) supplemented with 20ng/ml rhEGF (STEMCELL Technologies). The cells were allowed to incubate at 37°C/5% CO_2 for a period of 3-5 days until sphere formation began. Once cells had formed spheres they were sexed, then dissociated using ACCUTASE™ (STEMCELL Technologies) and passaged for immediate experimentation or cryopreserved for downstream investigation. Sexing primers were as follows: mSRY:5'CATTTATGGTGTGGTCCCGTG3', 5' CTCTGTGACACTTTAGCCCTC 3', GAPDH 5' TGACCTCAACTACATGGT 3', 5'CAGTGGATGCAGGGATGA 3'.

Testosterone Treatments: 3 independent XX and XY primary mouse neural stem cell lines were brought to single cell suspension using ACCUTASE™ (STEMCELL Technologies), and $1.0x10^6$ cells were seeded into a 10cm non-adherent culture dish. The cells were suspended in Complete Embryonic Neurocult™ Proliferation Media (STEMCELL Technologies) supplemented with 20ng/ml rhEGF (STEMCELL Technologies) and a single addition of TP yielding a final concentration of 20nM Testosterone Propionate (Sigma) on day 1, or the corresponding volume of DMSO vehicle (final concentration of $1.3x10^{-4}$%)(Sigma). The cells were allowed to replicate for a period of 5 days in each media type prior to being prepped for RNA extraction or passaged. After a 5 day growth period a subset of cells that were not used for RNA extractions were brought to a single cell suspension and $5x10^5$ cells were seeded onto 10cm non-adherent culture dishes. These cells were suspended in Complete Embryonic Neurocult™ Proliferation Media (STEMCELL Technologies) supplemented with 20ng/ml rhEGF (STEMCELL Technologies), without the addition of TP or DMSO. After an additional 5 days these cells were prepared for RNA sequencing.

RNA extraction: After 5 days of growth the NSCs in TP and DMSO were pelleted and washed 1x in PBS and then re-pelleted. The washed pellet was re-suspended in Buffer RLT-Plus (Qiagen), and then RNA was extracted following the standard protocol for the RNeasy® Plus Mini Kit (Qiagen). Post extraction the RNA was subjected to DNase treatment for 30 min using the TURBO DNA-free™ (Ambion). RNA integrity was assessed using the Agilent RNA 6000 Nano Kit (Agilent Technologies) following standard protocols at the UCLA Genotyping and Sequencing Core. All RNA samples that were used in these studies had RIN values greater than 8.0.

Library Preparation and RNA Sequencing and Expression Analysis: 1ug of total RNA from each sample was submitted to the UCLA Neuroscience and Genomics core (UNGC) for cDNA library preparation. Unpooled cDNA sequencing libraries were prepared using TruSeq Stranded Total RNA with Ribo-Zero (Illumina) following standard protocols. 8, 4 and 6 libraries were multiplexed and sequenced on an Illumina HiSeq 2500 next generation sequencing machine on three different runs. Sequencing reads were aligned using TopHat (v2.0.8b)[26] to the mouse genome (mm9) and Ensembl Mus Musculus GTF file (Mus musculus NCBI37). Each sample had an average of 36,067,841, 35,519,612 and 55,760,848 unique reads mapped with a frequency greater than 80.11%, 89.82% and 88.73% being successfully aligned to the reference genome. Cufflinks (v2.1.1) was used to find gene expression per sample and Cuffdiff from Cufflinks was used to find differential expression. A false discovery rate of 0.10 was implemented as the statistical analysis used to determine significance of differential gene expression depending on whether the uncorrected p value was greater than the FDR after Benjamini-Hochberg correction

for multiple-testing. Each comparison included 6 samples at two different conditions. Conditions were equally distributed among the three runs.

Quantitative-PCR Validation: 1µg of total RNA that had been previously extracted for RNA-sequencing was converted to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) following manufactures protocol. Post conversion, the final volume of cDNA was diluted 1:4 using nuclease-free water and pooled. Quantitative-PCR reactions were prepared using the PowerUp™ SYBR™ Green master mix (Applied Biosystems) following manufactures protocol, with the addition of 3ul of diluted cDNA and the appropriate forward and reverse primers for the gene of interest. All qPCR reactions were ran and analyzed on a Bio-Rad CFX-Connect Real-Time System. Cq values for control sample and testosterone exposed samples were normalized to Cq values for the housekeeping gene Beta-2-Microglobulin (B2M). Fold changes were determined by subtracting the experimental testosterone groups Cq from the Cq of the corresponding gene in the control group. Each analysis was run in technical quadruplicate for both the control and testosterone samples followed by a Student's t-test to determine statistical significance of fold-change for each tested gene.

Pathway analysis: The 103 genes that were found to be sexually dimorphic prior to hormone exposure were all subjected to pathway enrichment using DAVID bioinformatics software. The top 10 most statistically significant enrichments were tabulated using the biological process software feature. To determine unique pathways that were downregulated in each sex post-hormone treatment, algorithms from the Broad insitutues GeNETS software (PPI, ConcensysPathDB, GeNets Metanetwork V1.0, Geo, Achillies, Blast and CLIME) were

implemented for the top 200 most down regulated genes in each sex. For the mutually upregulated pathways both DAVID bioinformatics software and algorithms from the Broad institute's GeNETS software (PPI, ConcensysPathDB, GeNets Metanetwork V1.0, Geo, Achillies, Blast and CLIME) specifically the Metanetwork V1.0 were used in that analysis.

Discussion: Determining the mechanism by which gonadal hormones shape the developing brain and organize the central nervous system remains to be fully elucidated. Using an *in vitro* system, we sought to better understand the effect of testosterone on the transcriptome of the cells responsible for generating the central nervous system, eNSCs. We initially determined that gene expression between XX and XY eNSCs was sexually dimorphic, and 103 transcripts were uncovered as displaying differential expression (FDR=0.10). These basal sex differences appear to be enriched in pathways most involved in both positively and negatively regulating cellular proliferation. This is corroborated by several recent studies that demonstrated that gonadal hormones can affect NSC proliferation [14, 27]. Therefore, we provide evidence here that basal gene expression differences between XX and XY cells may contribute to differences in proliferation of NSCs in the absence of testosterone.

The gene expression differences in eNSCs identified here are the first documented cases of sexual dimorphism in gene expression between the sexes at this early stage within cells that comprise early CNS development. Unlike other studies that have focused on sex differences in the brain caused by genetic makeup [6, 21, 28, 29], our approach used a very specific cell type as opposed to gross brain tissue, as the latter may lack the sensitivity to observe nuances in gene expression, which may be masked by heterogeneous cell populations. This limitation has been observed when looking at DNA methylation in the brain, as it was found that global profiles are

striking different between individual cells and that of heterogeneous cell populations within whole brain tissue [30]. These findings also allude to the possibility that sex differences are fluid, and can be variable over developmental time and specific to regions of interest.

After determining that eNSCs show sexual dimorphism in gene expression, we wanted to determine if these cells also responded differently to the exposure of testosterone. Again, using a complete transcriptomic approach of RNA-sequencing, we determined that testosterone has differential effects on embryonic neural stem cells depending on sex chromosome composition. We found that upon exposure to testosterone XX NSCs differently expressed over 2800 transcripts, whereas in XY cells nearly 800 transcripts became differentially regulated (FDR=0.10). The effects of gonadal hormones on these cell types are just beginning to be uncovered, as such; this is the first documentation of global gene changes in eNSCs as a result of testosterone exposure during a developmentally relevant time in gestation [31]. It appears that TP on a XX background uniquely downregulates pathways involved in potassium channel signaling, but, on an XY background pathways involved in ERRB1 internalization are uniquely down-regulated (Fig. 3-5 C,D). Some of the effects of TP are also mutual between sexes. We identified 616 transcripts that were mutually upregulated upon active testosterone exposure. Interestingly these upregulate transcripts were most enriched in pathways that were involved in gene transcription, regulation, and genomic architecture in both XX and XY eNSCs (Fig. 3-6B). This finding adds to the growing body of literature demonstrating that TP can indeed cause increased proliferation events in this cell type. Since there are variable outcomes in gene expression after TP exposure depending on sex chromosome complement, inherent epigenetic programming may be involved which has set the stage for differential response to such hormones in each sex.

By exposing XX eNSCs to TP, we were able to eliminate and masculinize 42% of the basal sex differences that were identified prior to hormone treatment. These findings indicate that TP can elicit masculinizing events in this cell type, again adding an unknown component of androgen function within the cells abundant during early stages of brain development. The masculinizing effects of testosterone on the brain when administered to a female rodent during development, or at birth have been extensively documented [2, 32] however, the mechanisms of how such effects are maintained into adulthood still remain unclear. We also found that 25% of basal sex differences were completely feminized on a XY background post-TP exposure, an unexpected finding, as the feminizing effects of testosterone are not often researched. This finding is of great interest as it opens the possibilities for a sex-specific regulation of dimorphic genes in embryonic neural stem cells as a result of hormone exposures at various time points in development. For humans *in utero* testosterone exposure can have both feminizing and masculinizing effects depending on sex, as seen in our neural stem cell model. Research has shown that despite high/above average levels of testosterone during development, XY males with congenital adrenal hyperplasia (CAH) show reduced male-typical personality characteristics [33] and reduced visuospatial performances [34, 35] a male dominated strength. Females with CAH typically display the reverse in these personality [33] and cognitive [34, 35] abilities, shifting them towards a more male-typical pattern. These human observations raise the possibility that the timing and dose of TP exposure is critical for establishing male-typical neural outcomes in development.

As our goal was to better understand hormonal organization, we next investigated if gene expression in the daughter cells of the parentally exposed eNSCs maintained long-term gene expression changes. We determined that testosterone was unable to cause maintained

transcriptional differences in the daughter cell linages, in the absence of testosterone. While the activational effects of testosterone on the transcriptome are robust in both sexes, it appears that these differences revert back to near baseline if TP is removed from downstream cellular lineages.

The role of testosterone in masculinizing the rodent brain is traditionally thought to be less significant when compared to estradiol, the primary masculinizing agent of the rodent brain. But strong evidence has shown that mice lacking a functional androgen receptor (Tfm) are not completely masculinized in numerous adult behaviors, despite having functional estrogen receptors [36] Tfm mice display a de-masculinized pattern of copulation as compared to their wild type counterparts [37] which initially established the role that androgen receptor was required for complete masculinization of the rodent brain. Due to the fact that the cell type in this particular study is α/β estrogen receptor negative, as well as negative for P450 aromatase, it seems likely that the dramatic transcriptional and epigenetic changes that ensues within eNSCs post-TP exposure is mediated by action on the androgen receptor (AR), as this is the only transcriptionally detectable hormone receptor relevant to the study. However, additional experimentation is necessary to fully determine if the effects of TP observed in eNSCs are solely due to action on AR and not attributed to other elusive mechanisms.

These findings in eNSCs raise a completely new role for testosterone action within the early cells of the CNS, and have led to the proposal of a mechanism by which hormonal organization is able to be maintained through adulthood. Testosterone is capable of eliciting large transcriptional changes in eNSCs; thereby affect the epigenetic machinery of the cell. By doing so, TP possibly causes alterations in DNA methylation and perhaps even an active de-methylation event via the activation of TET proteins, as our RNA-seq data identified over a 1

fold increase in TET2 mRNA expression in the presence of testosterone (Supplemental Tables. 2, 3). In addition to DNA methylation, histone residues may also be modified either by increasing or decreasing acetylation and methylation levels at specific sites. Collectively, these epigenetic changes could possibly be maintained within the daughter cells, thereby creating new populations of eNSCs that now contain a modified epigenome. Upon differentiation large epigenetic events occur, and it is not unfathomable that these TP induced modifications of eNSCs will ultimately affect the downstream epigenomes of both the differentiating neurons and astrocytes. These modified differentiated cells may now be "epigenetically primed" and once exposed to estradiol at later time points in development, may become fully masculinized and enable variable adult behavioral phenotypes (Fig. 3-8). This possible mechanism could explain how despite significant neurogenesis over development the effects of *in utero* testosterone exposure are maintained and able to alter adult behavior. Since all CNS cells arise from eNSCs, if these populations have been re-programmed via gonadal hormone exposure, during self-renewal and differentiation the epigenetic changes that occurred early in development are maintained over the life of the mammal.

Our proposed mechanism of TP action on the early CNS will require additional downstream work, however, the evidence that we have uncovered alludes to a strong possibility that genetic/epigenetic modifications in eNSCs could permanently affect downstream cellular lineages on many different measures. Additionally, the elucidation of these basal sex differences and the sex-specific effects of testosterone on both the transcriptome and epigenome will contribute greatly to a new focus of study within the fields of neuroendocrinology and behavioral neuroscience.

Figure 3-1: A) Experimental design to explore both the activational and organizational effects of testosterone exposure on undifferentiated embryonic neural stem cells harvested from E13.5-14 male and female C57/BL6/J mice. B) Immunofluorescence staining of Nestin (Green) in XX undifferentiated neural stem cells prior to differentiation, demonstrating the strong expression of this eNSC marker. Immunofluorescence staining after 21 days of differentiation, demonstrating that our eNSC are capable of giving rise to both neurons and astrocytes as demonstrated by the neuronal marker Tuj-1/β3-tubulin (Green) and the mature astrocyte marker GFAP (Red). C) Average transcript expression (FPKM) of the neural stem cell markers Sox2 and Pax6. D) Average transcript expression (FPKM) of cellular markers for neurons (β-III Tubulin, DCX) and astrocytes (GFAP, Glul) prior to and post 21 days of differentiation, in addition to the NSC marker Nestin, which shows dramatic decrease in expression within differentiated populations.

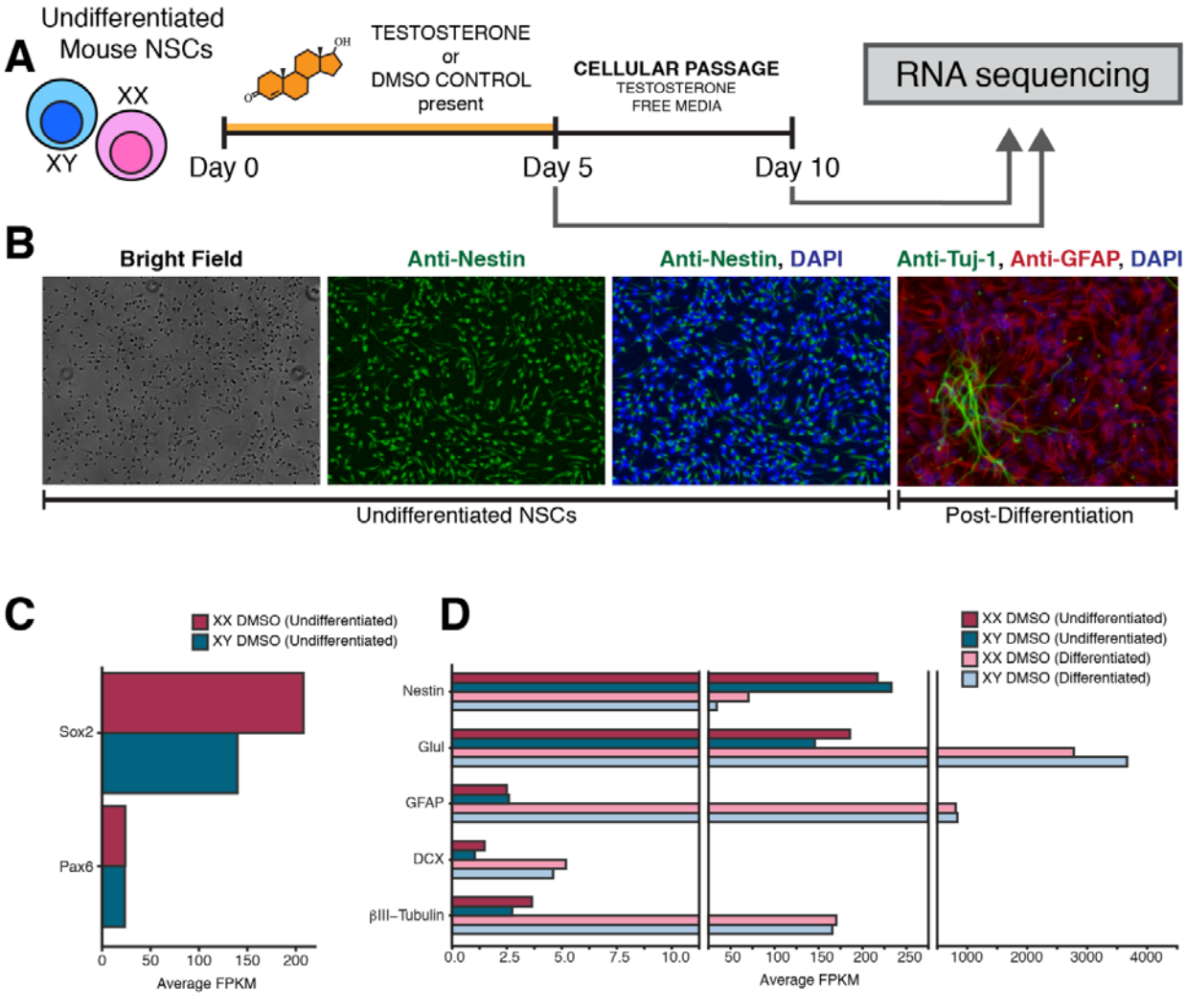
A
Undifferentiated
Mouse NSCs
XX
XY
TESTOSTERONE
or
DMSO CONTROL
present
CELLULAR PASSAGE
TESTOSTERONE
FREE MEDIA
RNA sequencing
Day 0
Day 5
Day 10
B
Bright Field
Anti-Nestin
Anti-Nestin, DAPI
Anti-Tuj-1, Anti-GFAP, DAPI
Undifferentiated NSCs
Post-Differentiation
C
XX DMSO (Undifferentiated)
XY DMSO (Undifferentiated)
Sox2
Pax6
0
50
100
150
200
Average FPKM
D
XX DMSO (Undifferentiated)
XY DMSO (Undifferentiated)
XX DMSO (Differentiated)
XY DMSO (Differentiated)
Nestin
Glul
GFAP
DCX
βIII–Tubulin
0.0
2.5
5.0
7.5
10.0
50
100
150
200
250
1000
2000
3000
4000
Average FPKM

Figure 3-2: A) A distribution plot representation of XX versus XY global gene expression (log2 FPKM). B) Represents the 103 differentially expressed genes (FDR=0.10) between the biological replicates of undifferentiated XX and XY embryonic neural stem cells, C) The top ten most significant biological process enrichments obtained from DAVID bioinformatics analysis software for the genes identified to be dimorphic in expression between XX and XY murine eNSCs.

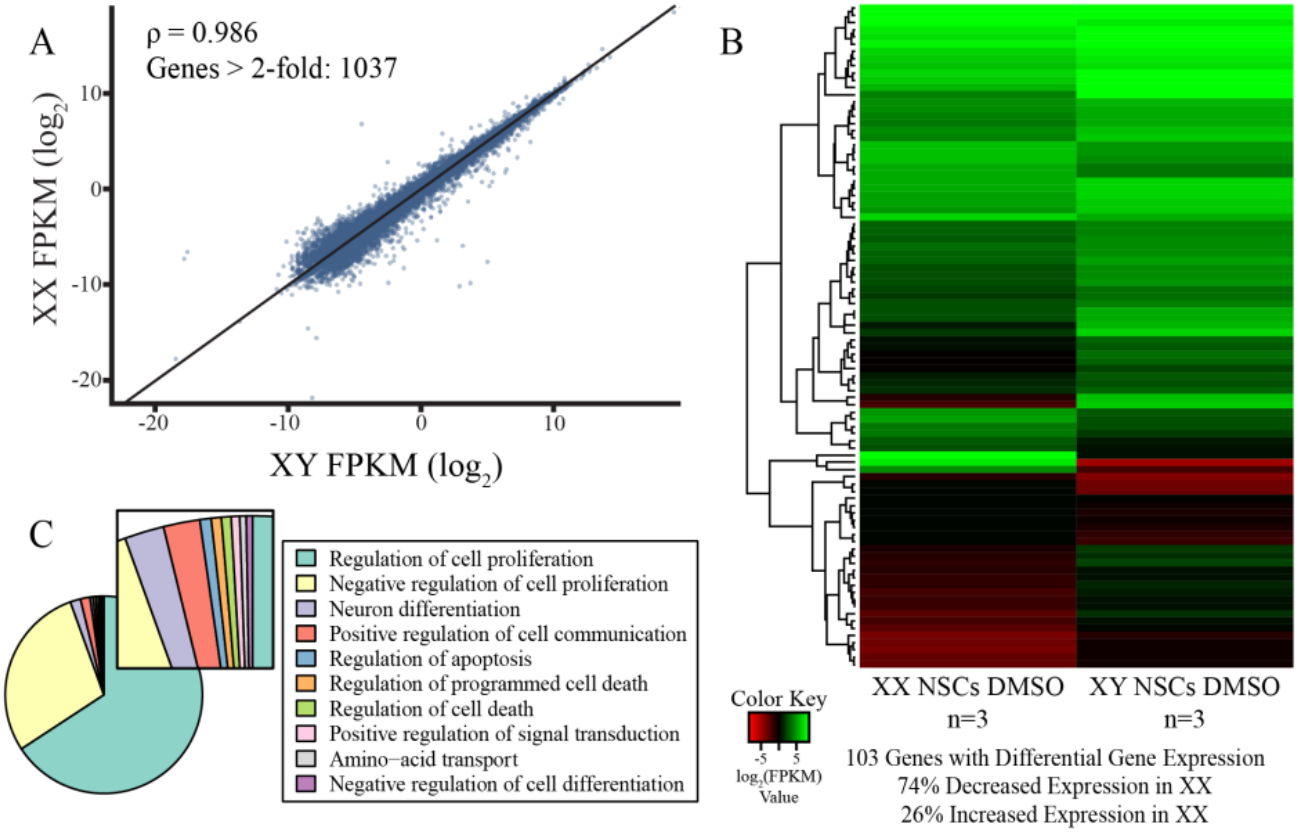

Figure 3-3: qPCR validation of 12/103 genes (Xist, Agt, Gbp2, Cox6a2, Cxcr4, Mmd2, ApoE, Ndgr2, Tnnt1, Pygm, Thrsp and Uty) that were identified by RNA-seq demonstrating basal expression differences between XX and XY eNSCs. Gene expression is represented as fold-change differences in XY baseline expression as compared to baseline XX eNSC gene expression levels.

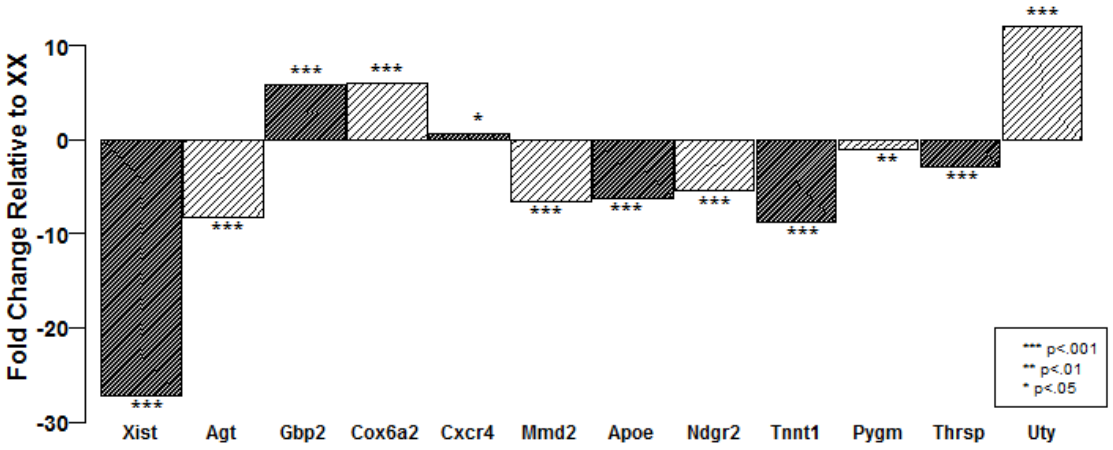

Figure 3-4: A,B) A distribution of global XX or XY baseline gene expression compared (Y-axis) to XX or XY gene expression in the presence of 20nM testosterone propionate (X-axis). C) Distribution ratios comparing the effects of chromosomal sex and testosterone on global gene expression of XX in TP/baseline gene expression (Y-axis), versus global gene expression changes due to testosterone of XY in TP/baseline gene expression (X-axis).

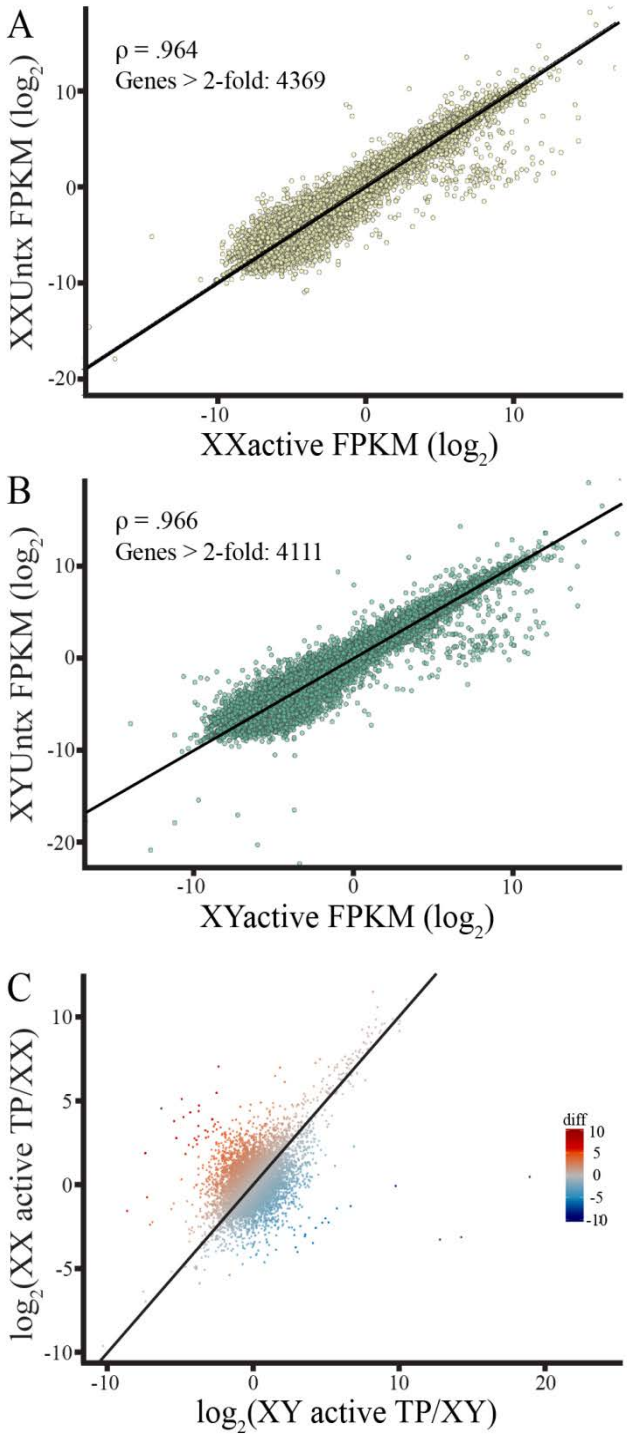

Fig 3-5: A) Heat map representation of the 2854 genes that were differentially expressed on an XX background after exposure to testosterone using an FDR =0.10, genes are clustered based on similar degrees of differential expression. B) Heat map representation of the 792 differential expressed transcripts on an XY background in the presence of testosterone using an FDR=0.10, genes are clustered based on similar degrees of differential expression. C) Genes involved in pathways responsible for downregulating of potassium channels which were uniquely affected in XX cells during TP exposure D) Genes involved in pathways responsible for ERRB1 internalization which were uniquely downregulated in XY cells during TP exposure

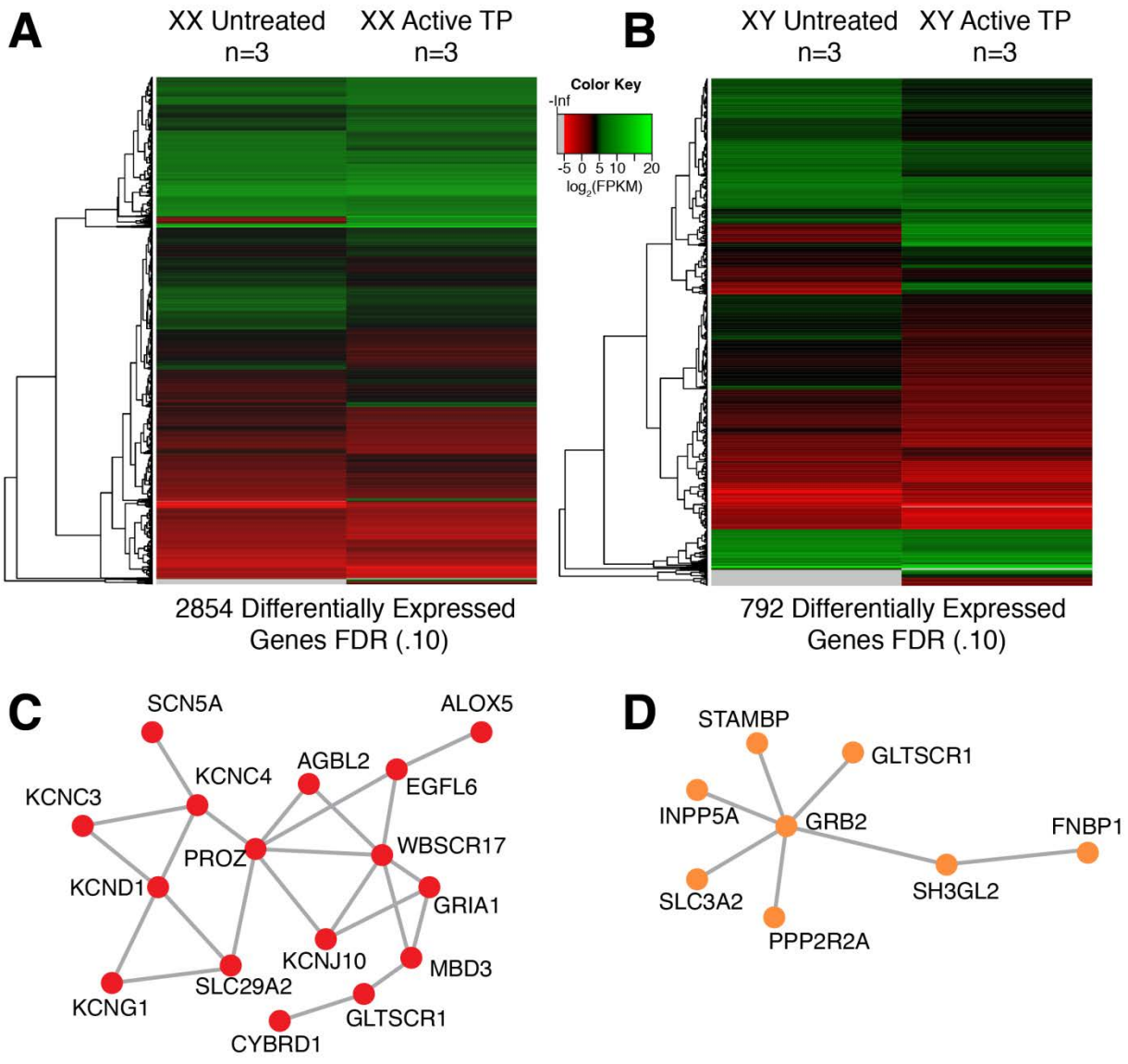

Figure 3-6: A) Dot plot representation of the delta $\log_2$ FPKM expression values for the 616 mutually affected genes on both XX and XY backgrounds post-TP exposure, with the top 10 most differentially affected genes highlighted. B) Up-regulated genes that are mutually shared between XX and XY in the presence of TP. The top three most up-regulated pathways are involved with nucleosome organization, nucleosome assembly, and chromatin assembly.

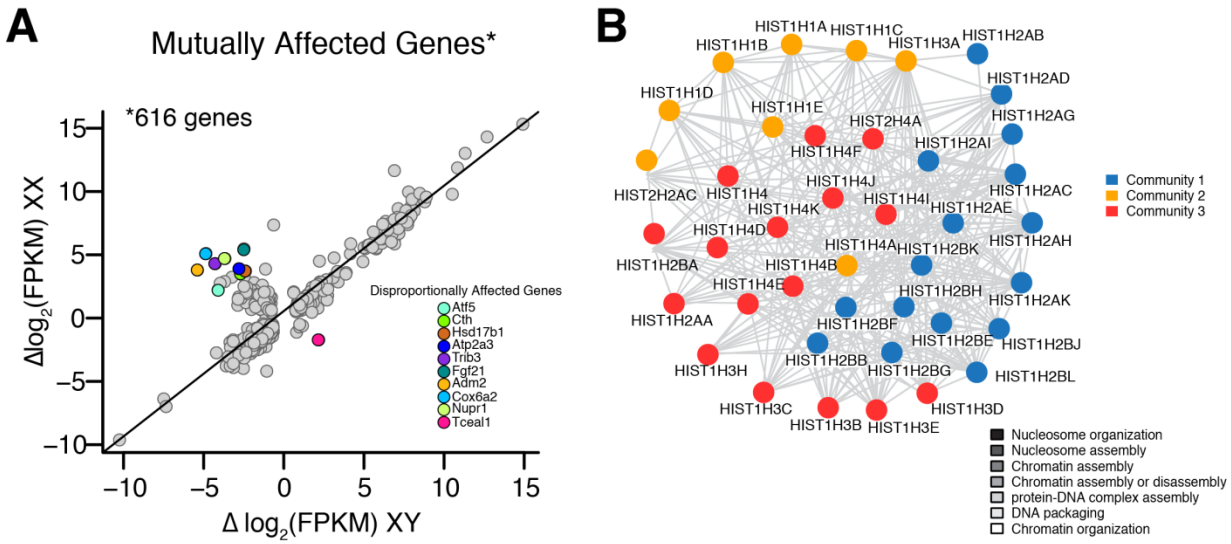

Figure 3-7: Circos plot representation showing the direction of masculinization or feminization of the 103 genes that were identified to be differentially expressed between XX and XY eNSCs at baseline. Genes connected by pink indicate that those genes either became or maintained a female typical expression pattern in the presence of TP. If the genes are connected by blue, this indicates that those genes either became or maintained a male-typical pattern of gene expression in the presence of TP.

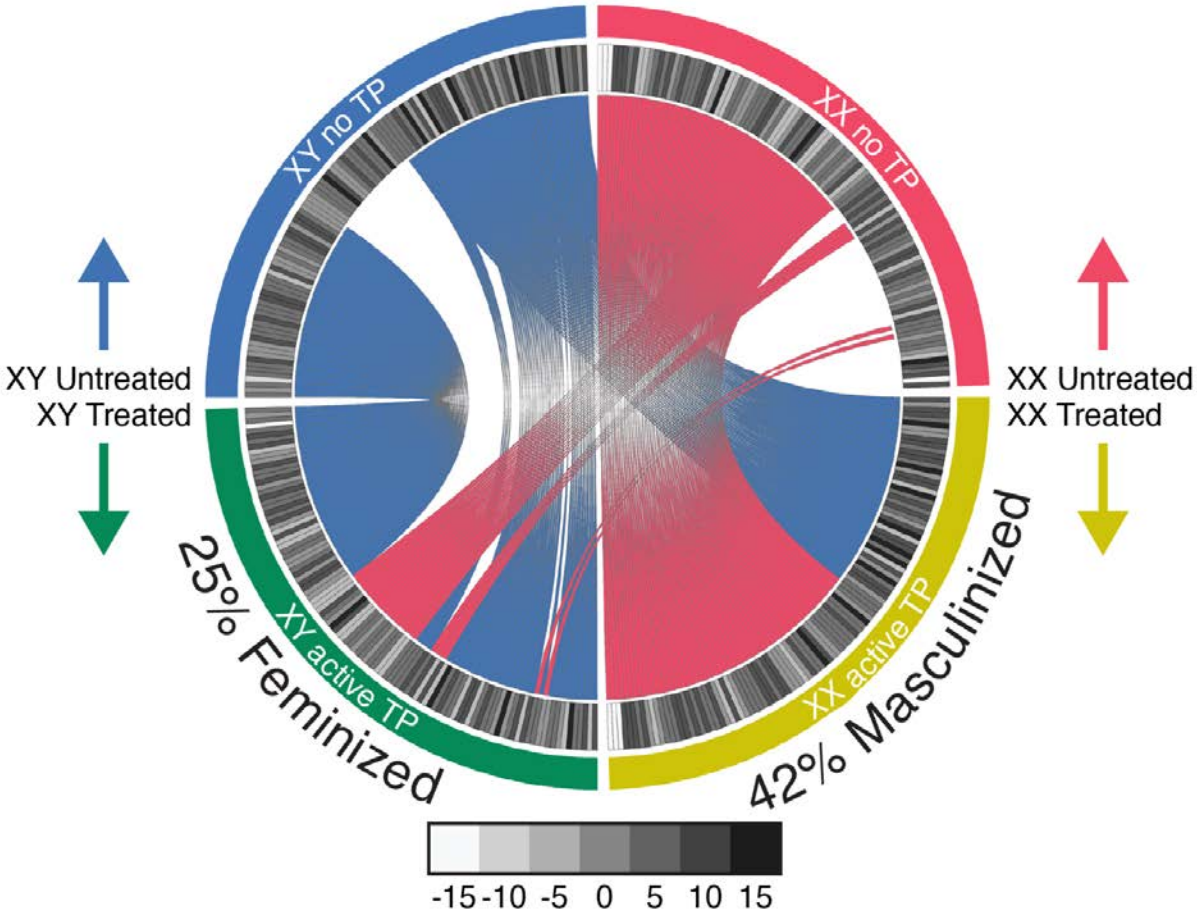

Fig 3-8: A proposed mechanism of early hormonal organization by which testosterone alters both the transcriptome and epigenome of neural stem cells, setting the stage for downstream effects in their differentiated progeny. Testosterone exposure results in large transcriptional affects that can alter epigenetic machinery and general DNA architecture. The exposure of TP may cause active DNA methylation changes and a remodeling of histone tale epigenetic regulators. After cellular division this altered epigenetic programming may be maintained thereby generating population of eNSCs which harbor re-programmed cellular memory and nuclear architecture. Upon differentiation it is likely that these epigenetic and transcriptional alterations effect both astrocytes and neurons, and "primes" them for complete masculinization or feminization during various time points of critical development.

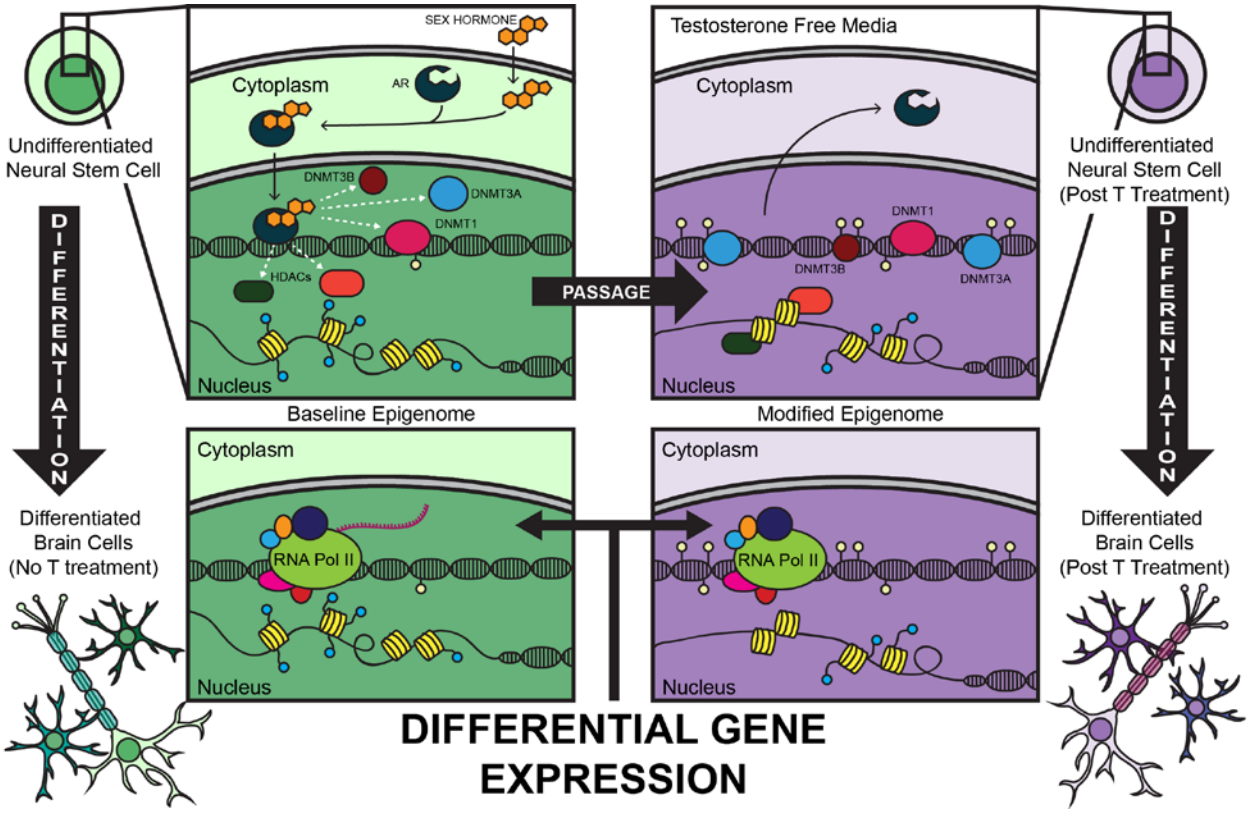

Table 3-1: Baseline Sex Differences and Post TP FPKM log_2 Expression Values

Gene	XX Baseline	XX + TP	XY Baseline	XY +TP		Gene	XX Baseline	XX + TP	XY Baseline	XY +TP	
1700008F21Rik	-3.61	-0.38	0.50	-2.82	¥	Mmp28	-2.52	0.23	1.22	-2.78	§
2700007P21Rik	4.34	6.57	5.79	4.82		Mthfd2	5.19	6.90	6.36	5.40	¥
AC103359.1	-Inf	0.71	-0.83	-1.55	¥	Myh8	-1.08	-2.08	-3.02	-2.16	
Abcc4	2.23	3.30	4.14	2.06	§	Nars	6.29	7.66	7.42	6.42	¥
Adm	4.32	5.48	5.38	4.45	¥	Nr2f2	0.12	-3.72	-3.28	-3.89	¥
Adm2	-1.44	2.35	3.29	-2.13	§	Nupr1	1.99	6.70	6.16	2.43	¥
Adora2a	-1.83	-0.82	0.44	-2.49	§	Odz4	-1.84	-0.29	1.54	-1.19	
Afap1l2	1.75	4.25	3.40	2.96	¥	Pck2	3.89	6.49	5.40	4.01	¥
Agap2	4.34	2.92	3.43	3.82	¥	Plekhb1	5.05	4.40	4.14	4.67	
Atf4	7.06	8.60	8.02	7.42	¥	Psph	5.05	7.10	6.37	5.34	¥
Atf5	4.88	7.11	8.02	3.86	§	Pygm	4.68	3.68	3.37	3.78	
Atp2a3	-3.43	0.45	-0.19	-3.01	¥	Rhbdd1	2.62	3.90	3.68	2.64	¥
Atp2b4	2.01	0.74	0.36	1.23		Sars	6.75	8.96	7.93	7.32	¥
Bdnf	-1.27	1.19	0.41	-0.37	¥	Sema4a	-1.03	-3.21	-2.86	-1.72	¥
Cadps	0.19	1.19	2.26	1.00		Sesn2	5.29	6.46	6.31	5.16	¥,§
Car3	6.81	5.69	5.65	6.42		Slc16a14	-1.87	0.07	0.02	-2.31	¥,§
Cd24a	5.65	6.40	6.57	6.00		Slc3a2	6.24	8.11	7.80	6.08	¥,§
Cd36	2.49	1.39	0.52	0.71		Slc7a1	2.61	3.91	5.71	4.34	
Cebpg	3.78	5.80	5.26	4.21	¥	Slc7a11	2.61	3.91	3.71	2.27	¥,§
Chac1	2.22	6.09	4.57	3.39	¥	Slc7a3	2.09	4.55	4.82	2.45	
Col28a1	-0.18	1.61	1.93	1.23	¥	Snhg12	5.12	6.95	6.87	5.25	¥
Cox6a2	1.61	6.70	6.07	1.19	¥,§	Sostdc1	-1.61	-0.12	0.80	-2.11	§
Cth	1.94	5.41	4.82	2.13	¥	Sv2a	3.32	2.39	2.24	2.32	
Cxadr	3.92	4.54	4.93	4.24		Tgif1	3.01	4.38	4.20	3.17	¥
Cxcr4	2.31	3.69	3.86	1.73	§	Thrsp	5.25	4.33	4.00	4.43	
Cyb5r1	3.28	6.17	7.03	3.30		Thy1	4.91	0.72	3.74	2.61	¥
Ddr2	1.85	3.82	3.78	1.84	¥,§	Tnnt1	3.45	2.12	1.73	2.19	
Eda	0.65	1.81	2.20	0.46	§	Trib3	0.72	5.02	5.08	0.75	
Eif4ebp1	5.63	8.14	7.10	6.35	¥	Ube2ql1	2.37	3.97	3.82	3.10	¥
Ero1l	3.69	5.29	5.40	3.51	§	Vegfa	4.60	6.59	3.93	4.48	
Fads3	1.28	2.47	2.63	1.34		Wars	5.57	6.39	6.66	5.55	§
Fam13b	2.52	3.17	3.72	2.27	§	AI464131	3.36	3.03	2.32	2.66	
Fbln5	-1.96	1.32	0.62	-1.88	¥	Agt	4.40	3.86	2.08	3.07	
Fbln7	1.25	2.93	3.51	0.67	§	Anxa6	3.97	4.04	4.93	4.61	
Gas1	4.96	6.05	6.01	5.39	¥	Apoe	9.30	9.52	8.18	9.89	§
Gbp2	0.36	1.78	2.76	0.40		Barhl2	-0.48	-0.01	1.27	1.17	
Gbp3	2.81	3.49	4.38	2.33	§	Creb3l1	1.40	1.51	2.67	-0.16	§
Glce	2.79	4.27	4.09	3.11	¥	Dll3	3.43	3.18	2.09	3.53	§
Gpr37l1	2.95	2.35	1.38	2.51		Dlx2	-1.77	-1.83	-0.07	-0.61	
Gpx3	3.46	2.41	2.34	2.51		Fam129a	-2.29	-1.37	0.06	-1.92	
Gsg1l	0.44	-1.85	-1.03	-1.42	¥	Fgfr3	5.66	5.06	4.69	5.40	
Hist1h1c	4.11	8.61	6.23	8.42	¥	Gpr17	4.12	3.80	2.52	3.74	
Hlx	1.90	3.07	3.15	1.72	§	Mmd2	4.90	4.54	3.45	4.66	§
Hrk	-1.84	-0.02	0.43	-1.65		Ndrg2	4.81	4.55	3.46	4.93	¥
Hsd17b1	-1.40	2.32	1.36	-1.07	¥	Scrg1	5.12	4.60	3.61	4.07	
Il6ra	-2.98	-1.76	-0.53	-2.94		Tmem28	-1.08	-0.39	1.27	-1.78	§
Jag1	3.59	4.96	4.74	3.83	¥	Usp18	-2.31	-2.10	0.18	-2.51	§
Klf4	2.09	4.21	4.00	2.13	¥	Xist	6.97	5.67	-4.49	-6.64	
Lars	4.80	6.42	5.91	5.11	¥	Uty	-Inf	-Inf	3.18	3.54	
Ldhb	7.68	6.98	6.96	7.02		Ube1y1	-Inf	-Inf	-0.44	-0.86	
Lhfpl2	1.80	4.12	3.92	3.13	¥	Kdm5d	-Inf	-Inf	2.95	3.30	
Mei4	-3.63	0.21	-1.03	-2.48	¥						

¥- Genes that were completely masculinized on a XX background

§- Genes that were completely feminized on a XY background

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Chapter 4:

Testosterone Induces Histone Modifications along with Transcriptional and DNA Demethylation within Embryonic Neural Stem Cells via Androgen Receptor Signaling

Abstract: Determining the mechanisms by which gonadal hormones organize and influence mammalian brain development has been a topic of much investigation over the past century. Studies have demonstrated the strong role that estrogens and to a lesser degree, androgens, have on masculinizing the brain and downstream adult behavior. Despite extensive investigation, limited explanations exist as to how these *in utero* exposures to gonadal hormones can have life-long effects on mammalian behavior in adulthood. Here we expand the role of androgen influence on the developing brain and demonstrate that testosterone has the power to significantly alter gene transcription and epigenetic programming via androgen receptor signaling. We determined that androgens cause sex-specific modifications to histone acetylation levels, and alter the acetylation levels in daughter linages as well. Most interesting, we determined that testosterone causes an active de-methylation event in eNSCs, a transmissible modification resulting in a decreased level of methylation within daughter cell populations. This effect is likely caused by an increase of de-methylation machinery such as Tet proteins, as we observe a dramatic increase of such expression in the presence of androgens. These findings have led to a more refined model as to how androgenic exposure during development contributes to initiating and possibly maintaining gonadal hormone brain organization.

Introduction: The exploration to understand ques that influence mammalian copulation, sex-typical behaviors and sex-biased neuropsychiatric disorders have been topics of much investigation over the past century. Early studies have demonstrated the influence of gonadal hormones on brain organization and sexual differentiation [1-3], while others have successfully shown the influence of genetic composition on such [4-7]. While the vast majority of early studies utilized rodents, increasing studies are now using human models to demonstrate the

effects of sex chromosomes and hormonal composition on brain development, behavior and sex-biased disorders, such as autism[8, 9]. Collectively, these studies and others have demonstrated the importance of hormonal and chromosomal contributions in regards to influencing to sex-differences in brain and behavior, yet clear molecular mechanisms that maintain these outcomes largely remains elusive.

More recent work has now begun to focus on other factors such as epigenetic influences that arise from a combination of hormonal exposure and individual genetic make up to explain some of the observed sex-differences. It has now been shown that testosterone and estrogen exposure on the developing brain can result in alterations of both DNA methylation and histone modifications [10-13]. These epigenetic modifications have been shown to largely influence rodent behavior, namely sexual, and have been proposed as the possible maintenance system for solidifying the life-long outcomes seen after early gonadal hormone exposure.

Most of the studies to date have used whole tissue analysis when measuring sex-differences in brain, but a growing body of evidence is now demonstrating the importance of using a homogenous population as opposed to gross brain tissue. Studies focusing on DNA methylation for example have shown that nuances in methylation can be greatly masked if specific cell types are not assessed independently[14]. The same appears true when measuring gene expression, as differences in such are largely spatial and cell specific within the mammalian brain; variations that otherwise may not be detected in a mixture of brain regions or cell types[15]. This important distinction has only moderately been taken into account when determine the effects of hormonal exposure and chromosomal sex composition within the brain. Some studies have shown the influences of testosterone and estrogens on specific types of

neurons and astrocytes [16-18], but these assessments are largely underdone and much is left to be obtained from this level of specificity, when assessing such influences.

To better formulate a molecular explanation as to how gonadal hormones influence brain development, our group has begun utilizing the foundational population of the central nervous system (CNS), neural stem cells (NSCs). While some groups have shown the effects of estrogens (E2) and testosterone (T/TP) on NSC proliferation and differentiation[19-23], our group recently determined that testosterone was capable of eliciting large transcriptional changes within NSCs in a sex-specific manner[24]. Until our group and others assessed some of the influences of gonadal hormone exposure on NSCs this topic had been of little discussion within the neuroendocrinology field. These cells may be the key to better understanding how hormonal organization and other brain sex-differences are initiated and sustained over the life of the animal.

To expand on earlier findings, our group wanted to determine if testosterone had other effects on NSCs in addition to altering global transcription. We wanted to determine if the observed effects were a direct result of testosterone signaling via androgen receptor (AR) or perhaps through alternative pathways. Here we present findings demonstrating that testosterone induced NSC gene transcription is facilitated in part by direct AR signaling. Most interestingly, we also demonstrated that in addition to altering gene expression, testosterone can modify epigenetic signatures such as histone tail acetylation and DNA methylation. We show that DNA methylation is actively reduced in NSCs during exposure to testosterone, a modification that is mediated through AR and is transmissible to daughter cell populations. These data are some of the first documentation of the role that testosterone has on altering epigenetic programming in

NSCs and expands a mechanistic proposal by which androgens may reprogram the early brain and generate masculine or feminine adult behavioral phenotypes.

Results:

Experimental Design: To assess the epigenetic effects of testosterone along with the role of androgen receptor, we utilized a similar experimental design as we have in previous RNAseq experimentation. Here we harvested multipotent neural stem cells from embryonic XX, XY and $XY^{AR-/-}$ from 13.5-14 C57BL6/J mice and followed the experimental timeline outlined in (Fig. 4-1A). After a growth period of 5 days, we stained for an accepted NSC marker, Nestin, which showed strong expression in both XY (data not shown) and XX cell types (Fig. 4-1B) demonstrating an accepted NSC staining profile. In addition to staining markers of eNSCs, these cells also give rise to both mature neurons and astrocytes, as demonstrated by the GFAP and Tuj-1 staining (Fig. 4-1B). Taken together, our immunofluorescence profile, gene expression data from previous experiments, and the differentiation potential, validate the multipotent characteristics of our experimental cell line. The difference between this design and the previous outlines were the final measures as our goals here were to assess target gene expression through qRT-PCR as opposed to global RNAseq along with histone acetylation and DNA methylation changes as a result of androgenic exposure.

Testosterone affects the Global Acetylation Pattern of Histone H3 in Daughter Linages of TP exposed NSCs:

Having uncovered large transcriptional changes in earlier studies between XX and XY eNSCs in the presence of androgen, we wanted to next examine if testosterone altered histone tail

acetylation, a strong marker of gene regulatory states[25]. Using an ELISA-based approach, we determined if testosterone affected the global percentage of acetylation on histone H3. We found that there were no significant differences in global H3 acetylation level between XX and XY embryonic neural stem cells at baseline (Fig. 4-2A). Unlike observations when looking at specific sites of tail acetylation (Fig.4-2B), we saw no significant changes in global H3 acetylation levels in either sex during active TP exposure, with XX cells being at 103% ($p=0.83$) and XY cells being at 106% ($p=0.72$) of baseline (Fig. 4-2A). Surprisingly however, we observe a dramatic increase in global H3 acetylation in both XX and XY daughter cells derived from the actively exposed parental linages. Specifically, Post-TP XX eNSCs show a 50% acetylation increase ($p<.01$) compared to baseline and Post-TP XY eNSCs were identified as have a 55% increase ($p<.01$) in acetylation compared to baseline (Fig. 4-2A). These data demonstrate there are late-emerging global hyper-acetylation events occurring within this progenitor cell population as a result of testosterone exposure.

Testosterone Causes Sexually Dimorphic Residue-Specific Alterations of Histone Acetylation levels:

Once it was established that testosterone was capable of altering global acetylation in eNSCs, a more site-specific analysis of lysine residue acetylation was conducted to determine if specific residues in both XX and XY eNSCs were differentially responsive to TP exposure. Using a western blot approach, we saw no significant changes in Lys9 of histone H2A, Lys5 of histone H2B, and Lys8 of histone H4 as a result of active TP-treatment in a XX eNSC background (Fig. 4-2B)(Fig 4-2F). In contrast, Lys9 on histone H3 was elevated 2-fold as a result of direct TP exposure ($p<.01$) (Fig. 4-2B)(Fig 4-2F). Furthermore, analysis of the post-treatment XX NSCs

demonstrated significant changes in acetylation levels at specific residues in the daughter cells of previously exposed eNSCs. Specifically, we report a 3-fold increase in Lys5 of H2A acetylation ($p<.05$), over a 1-fold decrease of acetylation on Lys5 of H2B ($p<.05$), maintained hyper-acetylation in Lys9 of H3 ($p<.01$) and a 1.5-fold increase in Lys8 of H4 ($p<.05$), all relative to the corresponding acetylation levels in post-treated control samples (Fig. 4-2C) (Fig 4-2F)

Unlike in XX cells, XY eNSCs showed significant changes in residue-specific acetylation on all core histones during active hormone treatment. Compared to baseline, we saw a 3.25-fold increase in acetylation on Lys5 of H2A ($p<.001$), a 2.5-fold increase in Lys5 of H2B ($p<.05$), a 50% reduction in acetylation levels on Lys9 of H3 ($p<.001$) and a 1.5-fold increase in Lys8 of H4 ($p<.01$) (Fig. 4-2D) (Fig 4-2F). Additionally, in the post-treatment daughter cells we also observed different outcomes than observed on a XX background. Again, compared to control post-treated cells, we see that acetylation levels of Lys5 of H2B revert back to baseline, Lys9 of H3 shows a 1.9-fold increase ($p<.05$) and Lys8 of H4 shows a 1.2-fold increase ($p<.05$). On Lys5 of H2A there is a ~50% reduction in acetylation levels, however these findings did not reach statistical significance ($p=0.1$) (Fig. 4-2E) (Fig 4-2F). Taken together, we see that active exposure to testosterone has both residue-specific as well as sex-specific effects on the acetylation abundance of core histone protein residues. Additionally, the effects of testosterone are not only transient, but can also affect acetylation levels in daughter cells from previously exposed eNSCs of both sexes.

Generation of AR Knockout Neural Stem Cells: To assess the influence of Androgen receptor (AR) in eliciting differential gene expression and downstream epigenetic effects we had to first isolate androgen receptor knock out (ARKO) eNSCs from E.13.5 mice. Using NSCs isolated

from B6.Cg-Aw-J EdaTa-6J +/+ ArTfm/J embryos we successfully generated NSCs with a nonfunctional AR. To demonstrate that AR was nonfunctional we allowed both XX and XY embryos to develop into adulthood where sexual differentiation was assessed; measured by both anogenital distance and internal reproductive structures. $XY^{AR-/-}$ show similar anogenital distance as compared to $XX^{AR-/+}$, as well as undescended and undersized testes, both are the traditional hallmarks of inability to respond to androgen (Fig. 4-3).

Testosterone Induced Decreases in Gene Expression is Modulated through AR: In our previous studies utilizing RNA-seq, we identified a pathway unique to a XY genetic background involved with the internalization of ERRB1 that was downregulated in the presence of TP[24]. While the response was strong and likely mediated through AR, given the absence of estrogen receptors in this cell population, we wanted to validate this claim using ARKO eNSCS. Following the experimental design (Fig 4-1A) RNA was isolated after 5 days of growth in the presence and absence of TP from XY and $XY^{AR-/-}$ eNSCs. qRT-PCR analysis revealed that the vast majority of downregulated genes identified using RNAseq were able to be validated using new cell lines and an alternate approach. We identified that *Fnbp1*, *Gltscr1*, *Grb2*, *Inpp5A*, and *Stampbp* showed significant downregulation on a XY background in the presence of TP. Using qRT-PCR *Gltscr1* and *Slc3a2* did not appear to show a decrease in expression as was identified in the global RNAseq screen (Fig 4-4). When this assessment was conducted using RNA isolated from $XY^{AR-/-}$, all significant downregulation that occurred in the wildtype XY lines was completely ablated. We see that *Fnbp1, Gltscr1, Grb2, Inpp5A, Sh3gl2, Scl3a2* and *Stampbp* showed no significant changes in expression in the presence of TP if AR was nonfunctional (Fig. 4-4). This is a strong indication that the gene expression downregulation is mediated though AR stimulated

pathways or direct regulation via AR binding to an ARE and not through other possible mechanisms.

Testosterone triggers a DNA De-methylation Event that is Maintained in Daughter Cell Linages of eNSCs and mediated via AR and possible increases of TET activity:

Since epigenetic modifications are beginning to be linked to the organization of the mammalian brain, we wanted to investigate if in addition to altering gene expression was testosterone able to alter global 5-methylcytosine abundance within eNSCs. We first assessed if there were baseline differences in global DNA methylation abundance between XX and XY eNSCs. Using an ELISA based approach, we determined that the global DNA methylation percentage in XX cells was 2.27% and 2.28% in XY eNSCs (Fig. 4-5B). These findings indicate that unlike gene expression, baseline global DNA methylation between the sexes is not sexually dimorphic in global percentage levels. After exposing the cells to testosterone for a 5-day period (Fig. 4-1A), we found that testosterone has a strong effect in reducing the levels of DNA methylation by roughly 50% in both XX and XY cells. We found that during TP exposure, global DNA methylation dropped to 1.06% ($p = <0.05$) on a XX background and 1.37% ($p = <0.05$) on an XY background (Fig. 4-5B). Most interestingly however, this reduction of DNA methylation was maintained within the daughter cell linages of TP exposed neural stem cells. We identified that the XX daughter cells from the previously exposed parental cells had a global DNA methylation percentage of 1.31% ($p<.05$) and the XY daughter cells had 1.26% ($p<.05$) (Fig. 4-5B). These findings indicate that testosterone actively reduces DNA methylation levels in embryonic neural stem cells, but most importantly demonstrates a long-term effect on altering the DNA epigenome in subsequent cellular linages after NSC replication from TP exposed cells.

We next wanted to determine if this effect was able to be ablated if AR was non-functional, similar to what was observed when gene expression was assessed in the presence of eNSCs with AR knocked out. When the same process was conducted in wildtype XY and $XY^{AR-/-}$ cells, we observed that at baseline both cell types have insignificant differences in levels of DNA methylation, 1.48% and 1.33% respectively (Fig 4-5C). Once the cells are exposed to TP we again see a significant >50% reduction in DNA methylation in wildtype XY cells ($P<.001$), however in cells with a nonfunctional AR we see no significant reduction in DNA methylation (Fig. 4-5C). These findings indicate that the active DNA demethylation is facilitated by activities downstream of androgen receptor activation.

Since it appears that DNA methylation is actively removed it seemed likely that this was mediated by enzymes that are known to facilitate such removal; Tet proteins. In our original RNAseq study we found that Tet2 was significantly upregulated in the presence of TP, and Tet1 and Tet3 trended towards significance as well. Using wildtype XY and $XY^{AR-/-}$ lines we determined if TP exposure could alter gene expression of the Tet proteins using both new cell lines and an independent method of qRT-PCR. We found that in the presence of testosterone, Tet1 ($P<.05$), Tet2 ($p<.05$) and Tet3 ($p<.01$) transcripts were significantly upregulated, validating and adding to findings of our global RNAseq study. When we assessed this using $XY^{AR-/-}$ none of the Tet transcripts showed a significant increase in expression, indicating again that functional AR is required to upregulate these particular proteins. Collectively, it seems that DNA methylation is actively decreased in the presence of TP via AR signaling which causes an upregulation of Tet enzymes, although additional experimentation will be necessary to confirm this complete proposal.

Methods:

Animal Care and Neural Stem Cell isolation: B6.Cg-Aw-J EdaTa-6J +/+ ArTfm/J mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and housed at the UCLA Animal Care Facility. Animals were maintained at 20°C with a 12-h light/12-h dark cycle, provided *ad libitum* with food and water. The study was approved by the University of California, Los Angeles (UCLA) Committee on Animal Research and was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. At eight weeks of age three dams and three males were mated separately to generate the biological triplicate basis, and the gestation was timed for 13.5 days. After the appropriate gestation time, the pregnant dams were humanely sacrificed, and immediately prepped for embryo extraction. Embryos were separated, followed by the removal of the brain which was then placed separately into dishes containing cold PBS/2% glucose. The ganglionic eminences from both hemispheres were removed and placed into 1.7ml Eppendorf tubes supplemented with cold PBS/2% glucose. This procedure was repeated for every embryo in each of the three dams following standard technical protocols [26]. Following isolation of the ganglionic eminence regions, the CNS tissue in each tube was triturated using a P-200 until no tissue clumps were visible prior to being passed through a 40µM cell strainer. After straining, all cells from each brain region were plated into 6-well culture dishes (Falcon) and suspended in Complete Embryonic Neurocult™ Proliferation Media (STEMCELL Technologies) supplemented with 20ng/ml rhEGF (STEMCELL Technologies). The cells were allowed to incubate at 37°C/5% CO_2 for a period of 3-5 days until sphere formation began. Once cells had formed spheres they were sexed and genotyped for androgen receptor mutants, then dissociated using ACCUTASE™ (STEMCELL Technologies) and passaged for immediate experimentation

or cryopreserved for downstream investigation. Sexing primers were as follows: mSRY:5'CATTTATGGTGTGGTCCCGTG3', 5' CTCTGTGACACTTTAGCCCTC 3', GAPDH 5' TGACCTCAACTACATGGT 3', 5'CAGTGGATGCAGGGATGA 3'. Androgen Receptor Mutation primers: 5' GACTACCCAAGTCCCCATAGC 3', 5' GGAGGT TACGCCAAAGGATT 3'. AR cycling steps: 95°C/3min, 95°C/30sec, 58°C/1min, 72°C/1min, (repeat steps 2-4/35 cycles), 72°C/2min, Hold. Post AR amplification, the PCR product was digested with MwoI (NEB) following manufactures standard restriction enzyme digestion protocol. Post-digestion the products were separated on a 2.0% agarose gel. Androgen receptor wild-type have products at 32bp, 36bp, 67bp and 145bp, Androgen receptor mutants have products at 32bp, 36bp and 211bp.

Testosterone Treatments: 3 independent XX , XY and $XY^{Ar-/-}$ primary mouse neural stem cell lines were brought to single cell suspension using ACCUTASE™ (STEMCELL Technologies), and $1.0x10^6$ cells were seeded into a 10cm non-adherent culture dish. The cells were suspended in Complete Embryonic Neurocult™ Proliferation Media (STEMCELL Technologies) supplemented with 20ng/ml rhEGF (STEMCELL Technologies) and a single addition of TP yielding a final concentration of 20nM Testosterone Propionate (Sigma) on day 1, or the corresponding volume of DMSO vehicle (final concentration of $1.3x10^{-4}$%)(Sigma). The cells were allowed to replicate for a period of 5 days in each media type prior to being prepped for RNA extraction or passaged. After a 5 day growth period a subset of cells that were not used for RNA extractions were brought to a single cell suspension and $5x10^5$ cells were seeded onto 10cm non-adherent culture dishes. These cells were suspended in Complete Embryonic Neurocult™ Proliferation Media (STEMCELL Technologies) supplemented with 20ng/ml

rhEGF (STEMCELL Technologies), without the addition of TP or DMSO. After an additional 5 days these cells were prepared for DNA and RNA extraction

RNA extraction: After 5 days of growth the NSCs in TP and DMSO were pelleted and washed 1x in PBS and then re-pelleted. The washed pellet was re-suspended in Buffer RLT-Plus (Qiagen), and then RNA was extracted following the standard protocol for the RNeasy® Plus Mini Kit (Qiagen). Post extraction the RNA was subjected to DNase treatment for 30 min using the TURBO DNA-free™ (Ambion), before downstream qPCR analysis was conducted.

Quantitative-PCR: 1µg of total RNA from each tested sample was converted to cDNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems) following manufactures protocol. Post conversion, the final volume of cDNA was diluted 1:4 using nuclease-free water and pooled. Quantitative-PCR reactions were prepared using the PowerUp™ SYBR™ Green master mix (Applied Biosystems) following manufactures protocol, with the addition of 3ul of diluted cDNA and the appropriate forward and reverse primers for the gene of interest. All qPCR reactions were ran and analyzed on a Bio-Rad CFX-Connect Real-Time System. Cq values for control sample and testosterone exposed samples were normalized to Cq values for the housekeeping gene Beta-2-Microglobulin (B2M). Fold changes were determined by subtracting the experimental testosterone groups Cq from the Cq of the corresponding gene in the control group. Each analysis was run in technical triplicate for both the control and testosterone samples followed by a Student's t-test to determine statistical significance of fold-change for each tested gene.

Global DNA 5-mC Quantification Assay: DNA methylation was assessed using the MethylFlash Methylated DNA Quantification Kit (Epigentek) following manufactures protocols. Assays were conducted using 100ng of DNA that was isolated using the gDNA™ MiniPrep kit (Zymo Research) following manufactures protocols. DNA was quantified prior to experimentation using a Qubit® fluorometer with the Qubit® dsDNA BR assay reagents. Samples were ran as three biological replicates in technical triplicate and final absorbance readings were subjected to a Welch two subject t-test to establish statistical significance for each comparison.

Global H3 Acetylation Assay: Global H3 acetylation was determined using the EpiQuik™ Global Histone H3 Acetylation Assay Kit (Epigentek) following manufactures protocols. Protein concentration of the nuclear extracts was quantified using a BCA assay, and 1.5µg of total protein was used form each experimental group. Samples were run using three biological replicates in technical triplicate and final absorbance readings were subjected to a Welch two subject t-test to establish statistical significance for each comparison. Acetylation levels were displayed as a ratio compared to the corresponding control H3 acetylation abundance.

NSC preparation for residue-specific acetylation assay: 3 independent XX and XY primary mouse neural stem cell lines were brought to single cell suspension using ACCUTASE™ (STEMCELL Technologies), and $1.0x10^6$ cells were seeded into a 10cm non-adherent culture dish. The cells were suspended in Complete Embryonic Neurocult™ Proliferation Media (STEMCELL Technologies) supplemented with 20ng/ml rhEGF (STEMCELL Technologies) and with the addition of a final concentration of 20nM Testosterone Propionate (Sigma), or the corresponding volume of DMSO vehicle (Sigma). The cells were allowed to replicate for a

period of 5 days in each media type prior to being prepped histone extraction. After a 5 day growth period a subset of cells that were not used for histone extractions were brought to a single cell suspension and $5x10^5$ cells were seeded onto 10cm non-adherent culture dishes. These cells were suspended in Complete Embryonic Neurocult^{TM} Proliferation Media (STEMCELL Technologies) supplemented with 20ng/ml rhEGF (STEMCELL Technologies) but without the addition of TP or DMSO. After an additional 5 days of growth these cells were prepared for histone extraction.

Histone extraction for residue-specific acetylation assay: $2x10^6$ cells from each NSC sample was collected and centrifuged at 1100 RMP for 10 min. The cellular pellets were washed 2X using DBPS and re-pelleted at 1100 RPM. The final washed pellets were re-suspended in 750µl lysis buffer (10mM HEPES (pH 7.9), 1.5mM $MgCl_2$, 10mM KCL, 0.34M sucrose, 10% glycerol, 1mM DTT, 1X protease inhibitor and 0.1% Triton-X). Cells were allowed to lyse on ice for 10min. Samples were then centrifuged at 4,500RPM for 5min at 4°C. Supernatant was removed and the pelleted nuclei were re-suspended in an additional 750µl of lysis buffer. Nuclei pellets were centrifuged at 4,500RPM for 5 min at 4°C followed by the aspiration of supernatant. Pellets were re-suspended and triturated in 50µl of histone extraction buffer (10% glycerol, 1.2% sulfuric acid, and 0.2% 2-mercaptoethanol). Samples were incubated on ice for 10 min then centrifuged at 13,000RPM for 10min at 4°C. Supernatants from the samples were collected and 12.5µl of tricholoroacetic acid (TCA) was added. Samples were vortexed and centrifuged at 13,000RPM for 10 min at 4°C. Supernatant was removed and mixed with 1mL of cold 100% ethanol then chilled at -80°C for 10min followed by centrifugation at 13,000 RPM. The ethanol

was aspirated then the samples were allowed to air-dry overnight. The dried pellets were stored at -80°C for downstream experimentation.

Histone quantification and normalization for residue-specific acetylation assay: Histone pellets were re-suspended in 100µl of ddH_2O. 30µl of suspended histones were quantified using the Pierce™ BCA Protein Assay (Thermo Fischer Scientific) following manufactures protocols. Standard curves for protein quantification were established using albumin standards (Thermo Scientific). Quantifications of samples were run in triplicate to establish a preliminary protein concentration. The 70µl of remaining protein sample was mixed with 4X LDS protein loading buffer and boiled for 10min in preparation for downstream PAGE analysis. Using the preliminary protein concentrations from the BCA assay, 0.9µg of protein from each sample group comparison (i.e XX Controls verses XX in TP) was loaded in duplicate onto a 15% SDS polyacrylamide gel and ran at 60V for 15min then 150V until appropriate protein separation occurred (~60min) in 1X Tris-Glycine buffer. The gel was washed in ddH_2O 3X for 5min each, then total protein was stained using SimplyBlue™ SafeStain (Invitrogen) for 1hr. The gel was de-stained overnight in ddH_2O and then imaged using a ChemiDoc MP Imaging System (Bio-Rad). Total protein densitometry for each lane was determined using ImageJ software (NIH) and statistical analysis of the pairwise groupings on each gel was determined using a Welch two sample t-test. Protein concentration was adjusted until the groups were statistically insignificant from one another, prior to western blot analysis used to determine acetylation levels.

Western blot analysis for the residue-specific acetylation assay: 0.9µg of sample was separated on a 15% SDS polyacrylamide gel and ran at 60V for 15min then 150V until proper protein

separation had occurred (~60min), in 1X Tris-Glycine buffer. The gel was then transferred onto a .45µM PVDF membrane using a Bolt® Mini Gel Tank Transfer Device (Thermo Fisher Scientific) at 30V for 3 hours in 1X Tris-Glycine buffer with the addition of 20% methanol. Membranes were blocked overnight at 4°C in 1X PBS with the addition of 5% NFDM. Post blocking, membranes were incubated overnight at 4°C with corresponding primary antibodies at a 1:500 dilution from the Acetyl-Histone Antibody Sampler Kit (Cell Signaling Technology). Membranes were then washed 3X 10min in PBST before being incubated 1hr in appropriate secondary antibody at a 1:15,000 concentration. Membranes were then washed 3X 10min in PBST prior to imaging. Pierce™ ECL Western Blotting Substrate was used at a 50:50 concentration to activate the HRP-conjugated secondary. Luminescence was measured using a ChemiDoc MP imaging system (Bio-Rad). Samples were analyzed using three biological replicates ran in technical duplicate. Band intensities were determined using ImageJ software (NIH) and sample values were subjected to a Welch two-sample t test to determine significance between experimental groups. Data is expressed as ratios of experimental samples compared to corresponding control samples.

Discussion: To date, the vast majority of studies that have assessed gonadal hormonal induced organization of the brain have concluded that estradiol is the primary masculinizing factor of the rodent CNS as opposed to testosterone[27]. These conclusions are based on the aromatization hypothesis, which has shown that testicular secreted testosterone is aromatized/converted into active estradiol locally within CNS tissue by the enzyme aromatase[28]. Despite very convincing evidence for the role of estradiol in organizing and masculinizing the rodent brain[29, 30], studies have shown that mice lacking a functional androgen receptor fail to become completely

masculinized in certain sex-typical behaviors, regardless of sufficient E2 response[31, 32]. These findings in ARKO mice, demonstrate a role for direct testosterone action on the developing brain, one that cannot be solely determined by the effects of estradiol.

To better understand the role of androgen action on the developing CNS our group utilized a neural stem cell model to test the effects of testosterone on eliciting changes within this fundamental cell type. Initially we determined that testosterone has sex-specific effects on NSCs and can significantly alter gene transcription in both sexes, but more so in an XX background[24]. These initial findings demonstrated that testosterone can have robust effects on gene transcription, but it remained unclear if androgen was capable of altering other cellular processes and through what mechanisms.

To expand on the role of androgen influence in the developing mammalian brain we first assessed whether androgen stimulation on eNSCs could elicit alterations in histone epigenetic signatures such as acetylation levels. We found that while testosterone exposure doesn't appear to cause a global change of acetylation levels on histone H3, there was a significant increase in acetylation levels within the daughter cells that were not exposed to TP. This finding demonstrated that while testosterone didn't seem to cause global changes in acetylation of H3, there were late emerging effects in downstream linages. This next prompted us to determine if there was residue or sex-specific differences in the response to testosterone with regard to histone acetylation. Much like our previous findings that identified sex-specific transcriptional effects of TP, we observed that there were also sex-specific histone modifications that occurred as well. It appears that testosterone can directly influence acetylation levels during active TP exposures, and this effect is more robust in an XY background as opposed to an XX. However, what is most interesting is what occurs in daughter cells that are generated in the absence of TP.

We see sex-specific responses to TP, but again we see that effects appear to be late emerging as was observed in the global H3 assays. Collectively, these findings indicate that androgen exposure on eNSCs can greatly alter histone modifications and results in modified acetylation levels of daughter cells that developed from parentally exposed NSCs.

Once we established that testosterone was able to alter histone modifications in addition to the transcriptional effects that were previous identified, we sought next to determine if the transcriptional effects were caused by direct AR signaling. Using AR knockout eNSCs, we determined if functional androgen receptor was required to downregulate a subset of genes that were found to be androgen responsive on an XY background. We determined that if AR is functional there are significant effects on gene expression. We see that genes such as *Fnbp1*, *Gltscr1*, *Grb2*, *Inpp5A*, and *Stampbp* are downregulated in wildtype XY eNSCs, but not in XY eNSCs with a nonfunctional AR. This finding has now increased our confidence that the mechanisms by which testosterone acts on altering gene transcription in neural stem cells is mediated through androgen receptor and not through other unknown pathways.

After determining that functional AR was required for gene transcription to be affected, we wanted to continue investigating the role of testosterone in altering other epigenetic marks. Utilizing a global assessment of 5-methylcytosine abundance we observe that TP exposure on both male and female eNSCs causes a dramatic decrease in DNA methylation. This reduction in DNA methylation was also maintained in daughter cells in the absence of TP, indicating that this reprogramming of eNSC DNA methylation was transmissible and persistent in downstream linages of previously androgen exposed cells. This finding is the first indication that testosterone exposure alters DNA methylation levels in eNSCs and highlights that androgens have the power to significantly alter epigenetic programming. This de-methylation event has also been observed

in specific brain regions post-estradiol administration [11], however the effect was attributed to arise from a dysregulation of DNMTs as opposed to active removal. Estradiol inactivation of DNMTs does not explain why methylation would be removed, as DNMTs function to add methyl groups to cytosine bases[33]. Knowing that ten-eleven translocases, or Tet proteins, actively remove DNA methylation, we sought to determine if these proteins were upregulated as a result of androgen exposure. Surprisingly, we identified that Tet1, Tet2 and Tet3 are all upregulated on an XY background, and again this effect was only possible if androgen receptor was functional. These findings indicate that as opposed to the proposal that DNMT activity caused a decrease in methylation at the gross brain level, we propose that it is the upregulation of Tet proteins that are responsible for these reductions in methylation.

Collectively, our findings have led to a proposal for an early mechanistic explanation as to how androgens influence early cells of the CNS, and possibly initiate and sustain hormonal organization (Fig. 4-6). We speculate that when eNSCs are exposed to testosterone there is dysregulation of many genes, but specifically a differential regulation of genes that serve as epigenetic regulators, such as the Tet proteins. Once these epigenetic regulators are stimulated they cause alterations to histones, but more importantly to DNA methylation. Specifically the Tet proteins actively remodel the methylome by reducing levels of DNA methylation at various sites across the genome, possibly facilitated by direct interactions with AR[34]. Once there has been a remodeling, the maintenance DNA methyltransferase, DNMT1, maintains this remodeled epigenome, thereby generating a new population of “primed” or masculinized eNSCs. Once these eNSCs differentiate it is possible that this reprogramming induced by testosterone causes the neurons or astrocytes to develop in a masculinized fashion once stimulated with E2 at later time points in development, once estrogen receptors are activated. While these findings are in

the early stages, we have provided sufficient evidence that testosterones actions via AR can greatly alter both transcription and the epigenome within eNSCs, expanding both the understanding and role of testosterone in the early developing mammalian brain.

Figures:

Figure 4-1: A) Experimental design to explore both the activational and organizational effects of testosterone exposure on undifferentiated embryonic neural stem cells harvested from E13.5-14 male, female and TfM male$^{Ar-/-}$ B6.Cg-Aw-J EdaTa-6J +/+ ArTfm/J. mice B) Immunofluorescence staining of Nestin (Green) in XX undifferentiated neural stem cells prior to differentiation, demonstrating the strong expression of this eNSC marker. Immunofluorescence staining after 21 days of differentiation, demonstrating that our eNSC are capable of giving rise to both neurons and astrocytes as demonstrated by the neuronal marker Tuj-1/β3-tubulin (Green) and the mature astrocyte marker GFAP (Red).

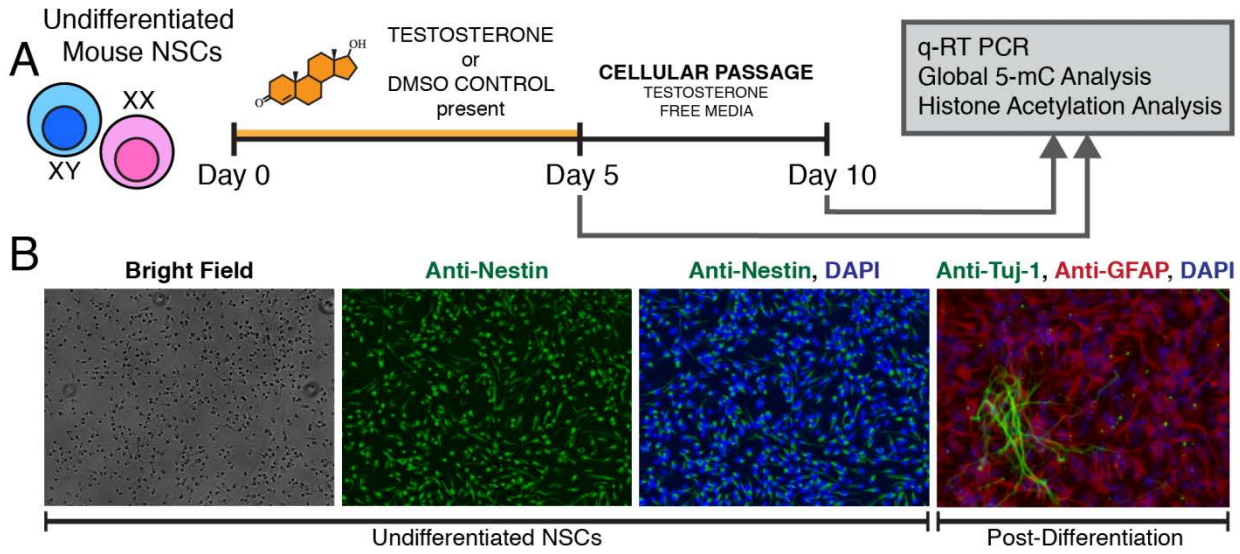


Figure 4-2: A) Global Histone H3 acetylation analysis of XX and XY eNSCs pre-TP exposure, during TP exposure and within the daughter cells post-TP exposure. B) Representative images of total protein assessment using Simply Blue stain which was used as the initial standardization for protein loading. Representative Western blots for XX and XX in TP measuring both core histone abundance and acetylation of specific residues. C) The same as panel B, except XY and XY in TP is being assayed. D) Representative image of total protein assessment using Simply Blue stain which was used as the initial standardization for protein loading. Representative Western blots for XX daughter cells post DMOS exposure and XX daughter cells post-TP exposure measuring both core histone abundance and acetylation of specific residues. E) The same as panel D, except measuring XY daughter cells post DMSO exposure and XY daughter cells post-TP exposure. F) Bar plot representation of residue-specific western blot analysis assessing acetylation abundance during active exposure to TP and within the daughter cells post-TP exposure relative to their corresponding control (DMSO) populations which have been set to a value of 1.

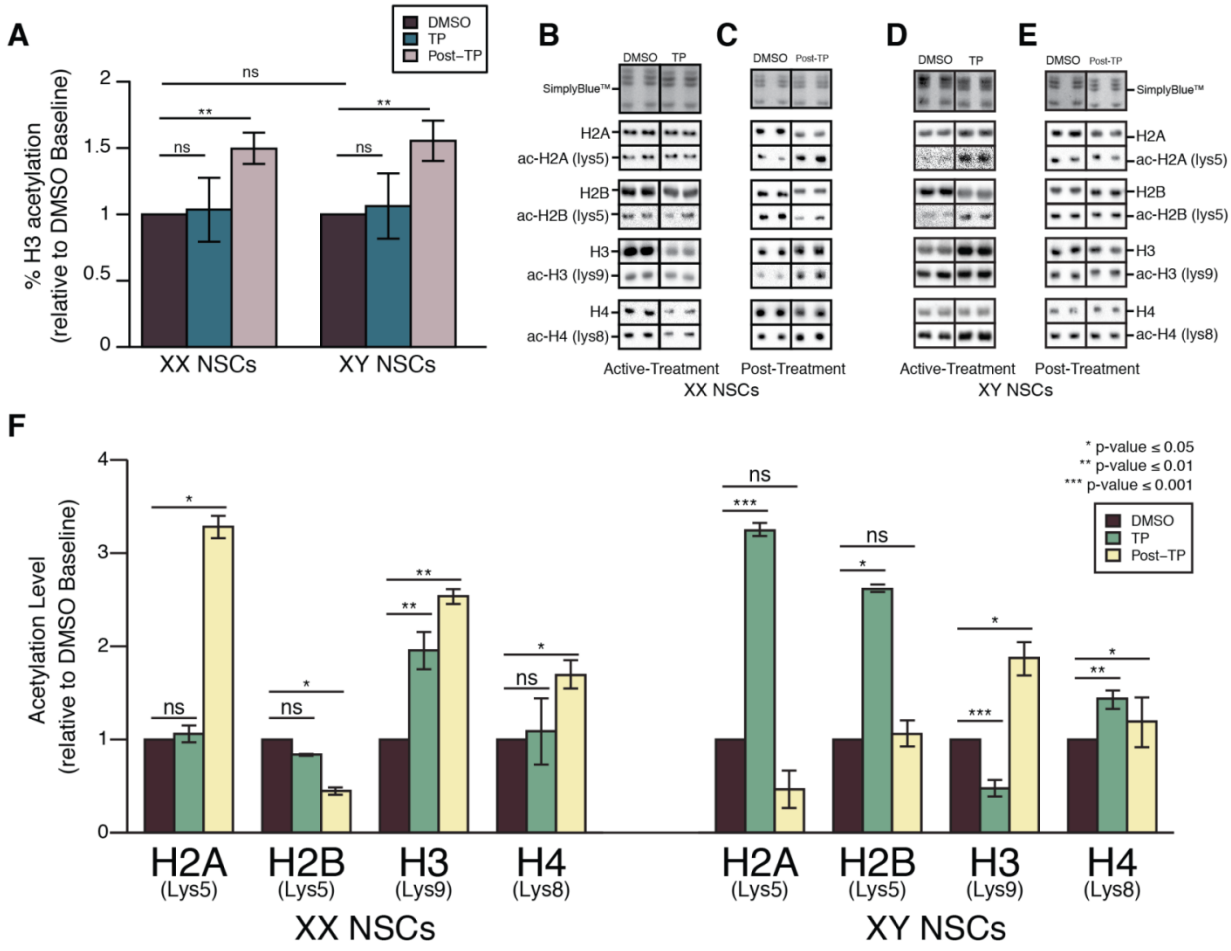

A
DMSO
TP
Post–TP
% H3 acetylation
(relative to DMSO Baseline)
ns
**
ns
**
ns
XX NSCs
XY NSCs
B
C
D
E
DMSO
TP
Post-TP
SimplyBlue™
H2A
ac-H2A (lys5)
H2B
ac-H2B (lys5)
H3
ac-H3 (lys9)
H4
ac-H4 (lys8)
Active-Treatment
Post-Treatment
XX NSCs
XY NSCs
F
* p-value ≤ 0.05
** p-value ≤ 0.01
*** p-value ≤ 0.001
Acetylation Level
(relative to DMSO Baseline)
H2A (Lys5)
H2B (Lys5)
H3 (Lys9)
H4 (Lys8)
XX NSCs
XY NSCs

Figure 4-3: 2 month old XX and XY (androgen receptor mutant/TfM) adult phenotype presentation: XX $AR^{+/-}$ phenotypically develops in wild-type fashion with normal ovarian function and typical external female genitalia. XY $AR^{-/-}$ TfM develops female appearing outward genitalia, with undersized and improperly descended testes, highlighting typical characteristics of a non-functional androgen receptor on a XY background.

Figure 4-4: Bar plot representation of a subset of genes that were downregulated on a XY background in the presence of testosterone-propionate. The genes that were responsive to TP exposure fail to be differentially regulated in a $XY^{AR-/-}$ background, demonstrating the role of AR in guiding this gene down-regulation.

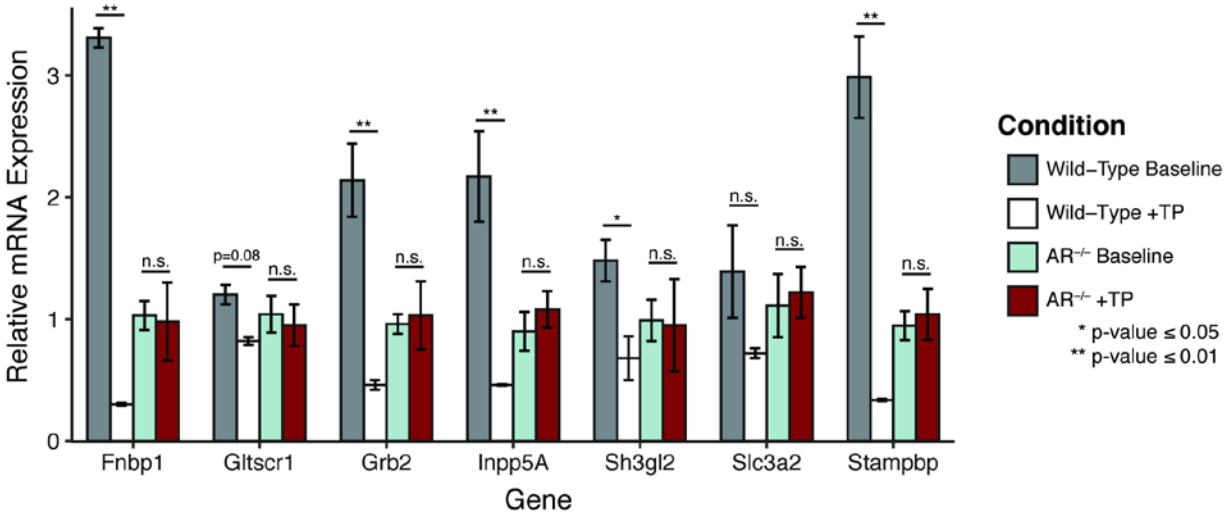
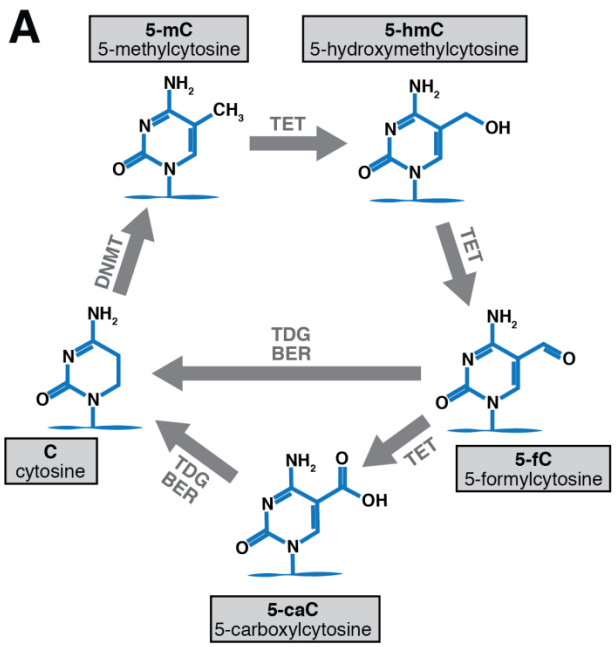
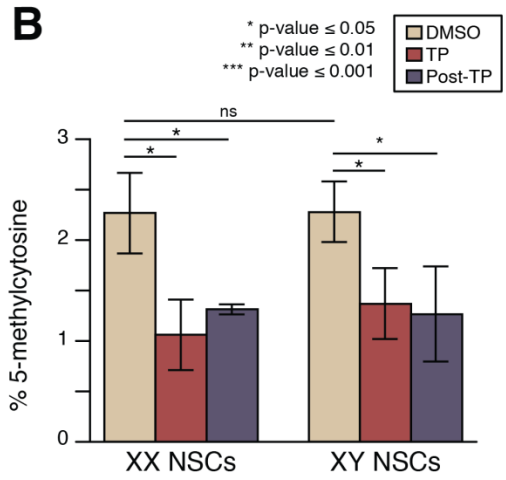
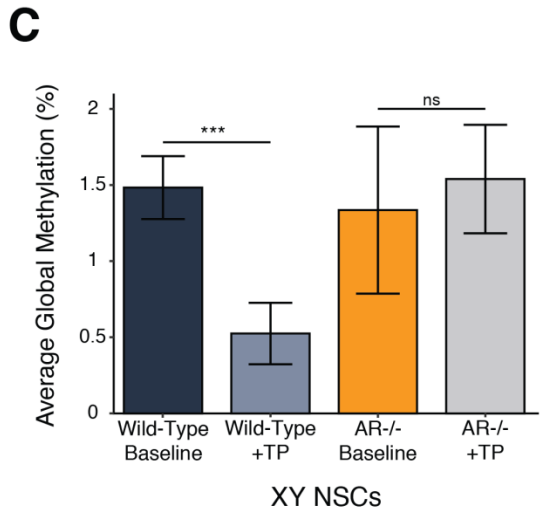


Figure 4-5: A) DNA methylation and De-methylation pathway overview B) Global 5-methylation analysis of XX and XY NSCs pre TP exposure, during TP exposure and within the daughter cells post-TP exposure. C) Global 5-methylation analysis of XY-wildtype and $XY^{AR-/-}$ at baseline and in the active presence of TP. D) q-RTPCR bar chart representation of Tet1, Tet2 and Tet3 relative mRNA expression at baseline and in the presence of TP using RNA isolated from XY-wildtype and $XY^{AR-/-}$


A
5-mC
5-methylcytosine
5-hmC
5-hydroxymethylcytosine
TET
TET
TET
DNMT
TDG
BER
TDG
BER
C
cytosine
5-fC
5-formylcytosine
5-caC
5-carboxylcytosine


B
* p-value ≤ 0.05
** p-value ≤ 0.01
*** p-value ≤ 0.001
DMSO
TP
Post-TP
% 5-methylcytosine
XX NSCs
XY NSCs
ns


C
Average Global Methylation (%)
***
ns
Wild-Type Baseline
Wild-Type +TP
AR-/- Baseline
AR-/- +TP
XY NSCs

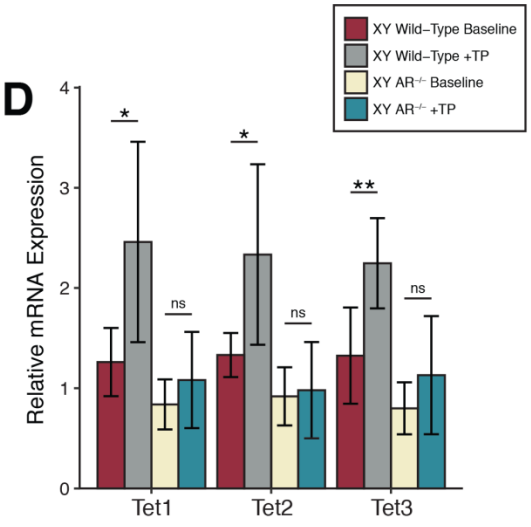
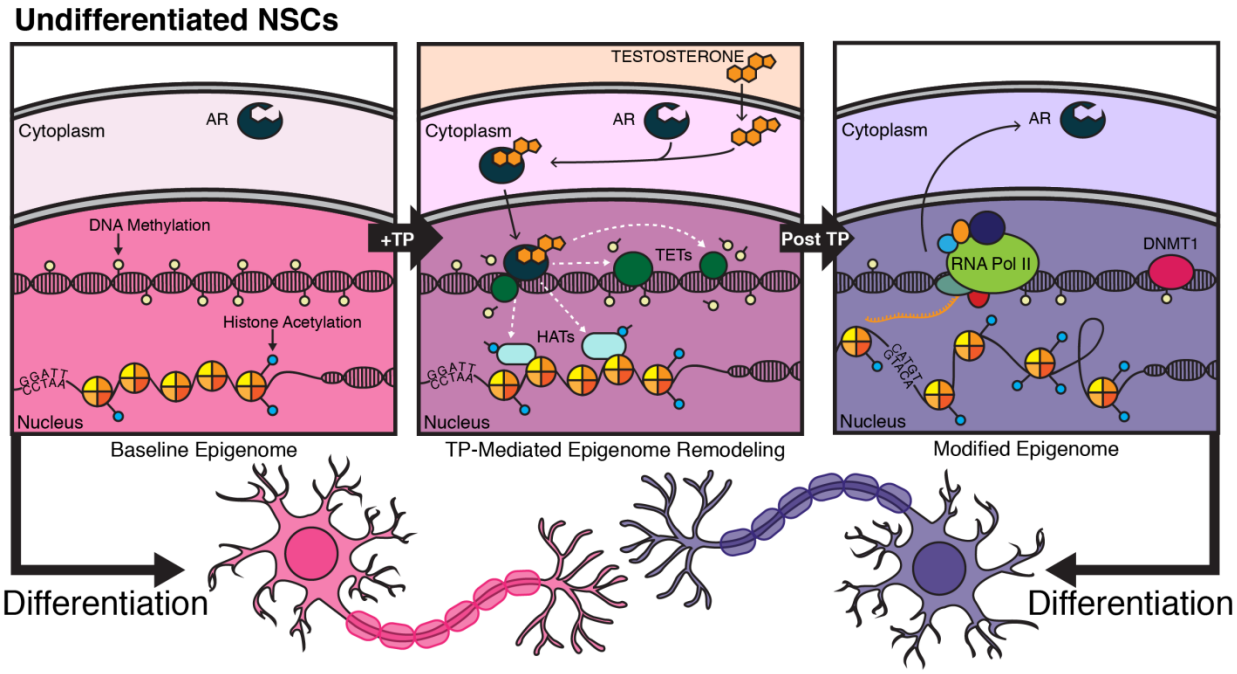

D
XY Wild–Type Baseline
XY Wild–Type +TP
XY AR-/- Baseline
XY AR-/- +TP
Relative mRNA Expression
Tet1
Tet2
Tet3

Fig 4-6: A proposed mechanism of early hormonal organization by which testosterone alters both the transcriptome and epigenome of neural stem cells, setting the stage for downstream effects in their differentiated progeny. Testosterone exposure results in large transcriptional affects that can alter epigenetic machinery and general DNA architecture. The exposure of T likely causes an active hypo-methylation event and a remodeling of histone tale acetylation abundance. After cellular division this altered epigenetic programming is maintained thereby generating population of eNSCs which harbor re-programmed cellular memory and nuclear architecture. Upon differentiation it is likely that these epigenetic and transcriptional alterations effect both astrocytes and neurons, and "primes" them for complete masculinization or feminization during various time points of critical development.



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Chapter 5:

Concluding Remarks and Research Contributions

To understand determinants of sexual development, sexual differentiation and factors that influence sexual behaviors, researchers have spent over a century developing theories, conducting research and raising eyebrows to answer such questions. In the early 1900's Frank Lillie postulated that fetuses of male cattle had substances/hormones circulating that enabled the masculinization of reproductive internal organs, and if exposed to female calves were able to masculinize theirs as well[1, 2]. This work arose from his studies focusing on freemartins, or infertile female calves with masculinized genital tract development; resulting from sharing a womb and blood supply with a male twin counterpart. These studies were some of the first to determine that male sexual development preceded female development and that circulating substances within a male fetus were potent and could result in the masculinization of genital tracts even in the opposite sex. This early work opened a new frontier and paved the way for later embryologist to begin investigating exactly what these substances where and how they were able to cause such dramatic developmental outcomes.

Building on the work of Lillie, around 1947 the skilled French surgeon, Alfred Jost, deduced that male testes secreted factors in addition to testosterone that caused the regression of Mullerian ducts, thereby masculinizing components of internal reproductive organs[3]. This unknown factor at the time was later identified to be anti-Mullerian hormone[4], hence its role in retarding the progression of Mullerian structure development. Over the course of a very successful and productive career Jost greatly expanded the understanding of early reproductive development and laid the foundation for the modern understanding of sexual differentiation of somatic tissue[5]. While Lillie and Jost greatly expanded the fields of embryology and reproductive development, the influence of testicular secretions on other organs such as the brain were still, and continue to be developing.

After the publication of the Phoenix et.al paper of 1959 [6], it became evident that the male testicular secretion of testosterone not only masculinized internal/external genital effectively, but also had profound consequences on rodent sexual behavior. As stated numerously through these various chapters, this work demonstrated that androgen exposure during *in utero* development could result in permanent effects on sexual behavior, effects that came to be known as organizational. These organizational effects are non-reversible and can subsequently lead to adult females displaying a defeminized pattern of copulation, and if supplemented with activational testosterone; male-typical sexual responses could be elicited. Although this work was foundational and has sustained scientific scrutiny as highlighted by A.P Arnold[7], additional components also influence this development of the male/female typical brains—genetic and epigenetic factors[8, 9]. Despite tremendous advancement in our understanding of the role of genes and hormones on shaping the mammalian brain, the mechanisms responsible for the initiation and maintenance of these outcomes are largely unknown. It is within this realm of expanding our mechanistic understanding of hormonal organization of the brain that I wanted to invest my research efforts, and as such, have added novel contributions to this ever evolving field.

Expanding transcriptional sex-differences and the role of androgenic influences on the development of the early central nervous system: As stated throughout, there have been significant expansions of the understanding of sex-differences within the mammalian brain that are independent of gonadal hormonal influence [8]. It has been shown on a small scale that sex-differences in gene expression arise prior to the onset of testicular secretions and complete gonadal formation, at least using murine models [10]. This particular study utilized whole brain

tissue isolated at E.10.5 of gestation, and found that around ~50 genes showed sexual dimorphic expression[10]. While this study expanded on the fact that genetic/transcriptional sex differences pre-date the onset of gonadal formation, the limitations within this study included non-homogenous cell populations and targeted gene microarrays. Our group wanted to investigate whether there were also basal transcriptional differences present in a specific population of cells within the developing CNS—embryonic neural stem cells (eNSCs). Using RNA sequencing, which provides a non-targeted global transcriptional analysis, we found that 103 genes were sexually dimorphic in expression at E13.5, after a false discovery rate (FDR) correction of 10%. This was the first documentation of such dimorphic gene expression between XX and XY NSCs to date. It appears that these 103 genes are enriched in pathways that pertain to various types of cellular proliferation, which is interesting as numerous studies have assessed the effects of sex and gonadal hormone exposure on proliferative capacity of both embryonic and adult NSCs[11, 12]. While it remains unclear as to exactly what these 103 genes collectively do within NSCs, we provided a gene list for which further exploration is warranted. In addition to demonstrating that the cellular foundations of the CNS inherently seem to express certain transcripts in a sex-specific fashion (See Chapter 3).

After we determined that eNSCs displayed sexually dimorphic gene expression patterns prior to the onset of gonadal hormone surges, we next sought to determine if these cells were responsive to androgenic stimulation. We found that by exposing eNSCs to a relevant dose of testosterone propionate (TP), gene expression levels became significantly altered and demonstrated a sex-specific response. It was found that 2854 genes became differentially expressed on a XX background, while 792 genes were differently expressed on a XY background. These findings highlighted for the first time that not only could androgens elicit

large transcriptional alterations, it did so in a sex-specific way, resulting in more robust effects on a female XX genetic background. Few others have demonstrated the androgen responsiveness of neural stem cells, but those that have, determined that such exposure could alter proliferation as well as perturb the differential potentials of the cells[13] (See chapter 2). To expand the understanding of action by which androgens affect gene expression we utilized eNSCs for which the androgen receptor (AR) was naturally mutated. We were able to determine that the mechanism by which these large gene transcription changes occur are downstream of AR signaling, as those cells with a nonfunctional AR show no alterations in expression of the select genes tested (See Chapter 4).

Upon closer examination we found the vast majority of genes that were affected on a XY background were also affected on an XX, indicating that there were sex chromosome independent effects of androgen exposure. Of these 616 mutually affected transcripts, there were similar trends, meaning that if TP caused gene expression to increase on an XY background it also did so on an XX. However, in addition to the mutual effects of TP regardless of genetic background, we identified a subset of those 616 genes that while mutually affected, were done so in opposite directions (See chapter 3). This finding may indicate that there are modular effects of TP and the response even within the mutually shared genes may be under the control of additional factors, such as epigenetic differences.

What we found to be most interesting in this study was when we examined what occurred to the original 103 basal sex-differences that existed between male and female eNSCs. It was determined that out of the 103 genes that displayed innate gene expression differences, 42% became completely masculinized/male typical on a XX background in the presence of TP. While this 42% shift was not a complete masculinization, this indicated to us that TP had the

power to alter basal differences on a female background, making them more male-typical. This was an exciting finding as, masculinized patterns of behavior and cognitive abilities are typically the most assessed measures when looking at the effects of androgen exposure during *in utero* development. What was also interesting is that 25% of basal gene expression on a XY genetic background became feminized/female-typical, in the presence of androgen. While this finding was surprising, it raises another interesting possibility in that the site/cell type of androgen exposure may be time and spatially sensitive. The feminizing effects of TP are rarely investigated, as this is the male-typical hormone, however, this topic is ripe for deeper analysis. Studies assessing individuals with Congenital Adrenal Hyperplasia (CAH) for example, have shown that males who develop in higher than average levels of TP actually show below typical-male scores on several cognitive assessments [14, 15] (See chapter 1). It appears possible that brain development is sensitive to both dose as well as timing of testosterone exposure, as supraphysiological levels of androgen do not generate supraphysiological levels of gene expression or human testosterone-sensitive cognitive abilities.

Collectively, the findings presented in chapter 3 are the first to demonstrate that murine embryonic neural stem cells display sexual dimorphic gene expression patterns, prior to the onset of gonadal hormone surges. Additionally, we demonstrate that not only can these cells respond to androgen they do so in a sex-specific manner; one that requires functional androgen receptor. While these findings are some of the first to assess global views of gene expression pre and post-TP exposure in eNSCs, significant work is still required to make biological sense of what these changes in gene expression do in the long-term span of brain development. Additionally, more functional studies will be required to completely characterize the exact pathway of action

downstream of AR signaling that enables such dramatic gene expression changes as a result of androgen exposure.

Revealing that androgen exposure on neural stem cells has dramatic and durable effects on altering the epigenome: While significant amounts of research have expanded the understanding of the contributions that genes and gonadal hormones have on shaping sex-differences and adult behavior within the brain; epigenetic influences appear to be the new frontier[16-18]. Once it had been established that eNSCs could respond to androgenic compounds in regard to altering gene expression, we next sought to determine if and how the epigenome was being affected. Proposals as well as studies have shown that gonadal hormones such as estrogens can modify histone epigenetic regulation and possibly contribute to development of stable brain sex-differences [19]. To expand on these theories, our group again utilized the neural stem cell model to investigate if testosterone propionate was also capable of eliciting histone modifications within this cell type.

We determined that TP exposure on eNSCs caused acetylation levels of histone tails to be significantly modified across all 4 histone groups; H2A, H2B, H3 and H4. Much like gene expression, these histone tail modifications were largely dependent on sex chromosome background, as we observed variable modifications of acetylation levels between XX and XY eNSCs as a result of TP. While the activational effects of TP exposure were not overly surprising since alterations in gene expression are frequently associated with dynamic shifts in epigenetic signatures on histone proteins[20], the sex-specific responses were (See chapter 4). What was most interesting when looking at the histone modifications triggered by androgenic stimulation was not so much the activational effects, but rather, the long term modifications that

we observed in the daughter cells that arose in the absence of TP. We observed that not only did androgen directly alter histone tail acetylation while actively present; it also influenced epigenetic tail signatures within downstream populations. This now raises the notion that exposures to androgen *in utero* could have long-term consequences on any responsive cellular population, long after the initial stimulations were received.

In addition to histone modifications, the most promising research to better help explain hormonal organization of the brain lies within our understanding of DNA methylation and possibly other DNA epigenetic modifications as well. Nugent and others in a recent publication [21], elegantly demonstrated that DNA methylation serves to maintain feminization of the brain, and that gonadal hormones, namely estradiol, could significantly reduce levels of such within certain brain regions. This study along with others from our lab[22], prompted us to next investigate if the DNA methylome of eNSCs was also sensitive to TP exposure.

We initially observed no sex-differences between the global levels of DNA 5-methylcytosine percentages between XX or XY eNSCs, indicating that at baseline the global levels of DNA methylation appear to be similar in nature. Once these cells were actively exposed to TP, there were dramatic reductions in the level of DNA methylation (>50%) in both sexes. This suggests that the response and possibly the driving mechanism for altering this reduction are similar regardless of chromosomal composition. What was most thought-provoking and possibly the largest impact of this work, was the demonstration that even once TP was removed these reductions in DNA methylation were maintained in downstream cellular progeny. These outcomes showed that these epigenetic alterations are transmissible to the daughter linages, thereby maintaining the effects of androgen exposure (See chapter 4). Using AR knockout eNSCs, we found that these modifications to the epigenome required functional

androgen receptor, and if AR was nonfunctional there were no significant reductions in DNA methylation. These findings are some of, if not the first, demonstrating that androgenic compounds can have profound and long lasting effects on eNSC DNA methylation which is modulated through downstream signaling post-AR activation.

Nugent et.al speculated that the reductions of DNA methylation they observed by administering estradiol to rodents was caused by reduced DNMT3A activity [21], however, we felt that this explanation was functionally difficult to explain. DNMTs serve to actively methylate, hence their name De novo methyltransferase, but they are not known to de-methylate [23]. When further exploring genes that were actively altered in the presence of TP, we found that Ten-eleven translocase-2 (Tet2) was significantly upregulated in both XX and XY eNSCs (See Chapter 3). This enzyme along with Tet1 and Tet3, unlike DNMTs, can remove DNA methylation from the genome and with Thymine-DNA glycosylase (TDG) can revert nucleic acids back to an unmodified cytosine[24]. On that premise, we wanted to validate if the Tet proteins were indeed upregulated by TP and if these upregulations were a result of AR function or though other unknown mechanisms.

We found that androgen indeed causes a significant upregulation of not only Tet2, but Tet1 and Tet3 in XY eNSCs with functional AR. When we assessed this measure in ARKO cell lines, this upregulation was ablated and neither Tet1, 2 or 3 showed any significant upregulation in the presence of TP, indicating again that this effect is modulated through AR signaling. This is a very exciting finding as it expands on how hormonal organization is perhaps initiated and maintained, but it also could be a possible way to manipulate DNA methylation if we uncover the target sequences that are poised for androgen dependent de-methylation. It may even be

possible that AR directly guides Tet proteins to regional sites across the genome, as recent studies have shown direct interaction between AR and Tet2 within prostate cancer cell lines[25]. In conclusion, our work using an eNSC model to expand the understanding of hormonal brain organization has contributed novel findings to the field, in addition to identifying new outcomes of androgen action on the developmental CNS. While the exact mechanics of hormonal brain organization still remain elusive even after our contributions, we feel that there are now new scientific paths to follow, enabling insight into how this phenomenon occurs, but more so how it is maintained over the life of the animal.

The End

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