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Role of DNA Methylation in Sexual Differentiation of Neurochemical Phenotype in the Brain

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by

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Under the Direction of Nancy G. Forger, PhD

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ABSTRACT

Sex differences in the brain underlie sex-specific behaviors and physiological processes, and may help to explain male- or female-biased neuropsychiatric disorders. Some of the best-studied neural sex differences depend on differential cell death in males and females, but other sex differences persist even if cell death is prevented. These include sex differences in stable patterns of gene expression, or what we refer to as the differentiation of neurochemical phenotype. The mechanisms contributing to sex differences in neurochemical phenotype are unknown, but epigenetic modifications, such as DNA methylation, control cell phenotype "decisions" throughout the body in developing animals. We recently discovered that expression of enzymes that place or remove DNA methylation marks peaks during the first week of life in the mouse brain and overlaps with the perinatal critical period of sexual differentiation. Thus, my over-arching hypothesis is that sex differences in DNA methylation early in life underlie sexual differentiation of cell phenotype. I tested this using a combination of techniques, including: pharmacological inhibition of DNA methyltransferases, siRNA knock-down of Ten-eleven-translocases, immunohistochemistry, pyrosequencing, and *in situ* hybridization. The results of this dissertation demonstrate that 1) neonatal inhibition of DNA methylation abolishes several sex differences in cell phenotype in the hypothalamus; 2) DNA methylation and demethylation both contribute to sex differences in the development of one cell type (estrogen receptor alpha, ERα) in the hypothalamic ventromedial nucleus (VMH) and arcuate nucleus, in a region-specific manner; and 3) sexual differentiation of cell phenotype in the VMH is not present at birth, but develops over the first few postnatal weeks and involves a developmental decrease in cell marker expression of *Tac1*, *Rprm*, and *Pdyn,* specifically in males. In summary, we demonstrate that neonatal DNA methylation and demethylation establish neurochemical cell

phenotype in a sex- and brain region-specific manner, providing the first studies demonstrating a mechanism by which sexual differentiation of neuronal cell type occurs.

INDEX WORDS: Sex Differences, Neurochemical phenotype, Epigenetics, DNA methylation, ERα, Development

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DEDICATION

To my mom and dad, your support and dedication to me and my siblings is immeasurable. Thank you for your sacrifices and for fostering a love of learning and curiosity about life and nature. To my brothers, your friendship keeps me happy and sane, and your jokes keep me humble.

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LIST OF ABBREVIATIONS

ARC arcuate nucleus

AVPV anteroventral periventricular nucleus

BNST bed nucleus of the stria terminalis

BNSTp principal nucleus of the BNST

DNMT DNA methyltransferases

ERα estrogen receptor alpha

HAT histone acetyltransferase

HDAC histone deacetyltransferase

hmC hydroxymethylcytosine

mC methylcytosine

MBD methyl-CpG-binding domain

MECP2 methyl-CpG-binding protein 2

mPOA medial preoptic area

NCOR nuclear receptor corepressor

PeN periventricular nucleus

POA preoptic area

SiRNA short/small interfering RNA

SNB spinal nucleus of the bulbocavernosus

SRC1 steroid receptor coactivator 1

VMH ventromedial hypothalamus

1 INTRODUCTION

1.1 Dissertation Overview

The human body comprises hundreds of diverse cell types that control different facets of physiology and govern our thoughts and behavior. Yet every cell stems from the same fertilized egg and contains the same genetic material: 22 pairs of autosomal chromosomes and 1 pair of sex chromosomes. This amazing diversity among cells carrying essentially the same DNA underscores the profound dependence of development on "epigenetics," literally, modifications "upon" the chromatin that cause lasting changes in gene expression without changing the underlying DNA nucleotide sequence.

Sexual differentiation (the differentiation of the initially bipotential embryo to become male or female) also depends on epigenetic mechanisms. This is most obvious when considering animal species without chromosomal sex. In some reptiles, for example, there are no sex chromosomes; genetically identical individuals may become either male or female (Charnier, 1966; Spotila et al., 1987; Deeming et al., 1988). Sex determination in painted turtles (*Chrysemys picta*), for instance, is the result of temperature differences during egg incubation, which cause epigenetic modifications to genes involved in hormone signaling (Radhakrishnan et al., 2018). Thus, *epi*genetic processes determine sex in this species. In mammals, males and females are not genetically identical: all female cells carry two X chromosomes, while male cells carry one X and one Y chromosome. However, as I hope to convince you in this chapter, epigenetic processes nonetheless play an important role in mammalian sexual differentiation, including that of the central nervous system.

Several of the best-studied sex differences in the brain are differences in cell number and are due to gonadal steroids, such as testosterone, acting early in life. One way that testosterone causes sex differences is by altering cell death; however, some sex differences persist when cell death is prevented, and many of these are related to cell phenotype. For example, males have more calbindin- and vasopressin-expressing cells in the bed nucleus of the stria terminalis (BNST), while females have more kisspeptin cells in the hypothalamus (anteroventral periventricular nucleus (AVPV)/periventricular nucleus (PeN)), and these sex differences persist in *Bax* -/- mice, in which developmental neuronal death is nearly completely eliminated (Forger et al., 2004; de Vries et al., 2008; Semaan et al., 2010; Gilmore et al., 2012). This suggests that neurochemical phenotype, rather than cell number, is sexually differentiated. Differences in neurochemical phenotype (essentially, long-lasting patterns of gene expression within a cell), are probably the most common type of sex differences in the brain, although the mechanisms driving their development are largely unknown. Given the role that epigenetics plays in establishing cell fate during development, the overarching hypothesis of my dissertation is that epigenetic processes early in life underlie sex differences in neurochemical phenotype. While there are different layers of epigenetic regulation, I focus on DNA methylation given its stability and specificity in modulating gene expression.

In a publication I co-authored during the first year of my PhD training, we found that inhibition of DNA methylation early in life abolishes sex differences in two cell types in the hypothalamus (estrogen receptor (ER) α and calbindin neurons), without altering cell death in mice (Mosley, Weathington et al., 2017). Thus, in Chapter Two of this dissertation we test 1) whether these sex differences depend on gonadal hormones, and 2) if so, whether the masculinizing effects of gonadal hormones require DNA methylation. We find that testosterone

treatment completely masculinizes the number of ERα, calbindin, and kisspeptin expressing cells in the mouse hypothalamus. Females administered a DNA methylation inhibitor concurrent with testosterone have ERα cell numbers in-between that of males and females, indicating that hormone-dependent masculinization was partially prevented. In contrast, kisspeptin masculinization was unaffected. Thus, we show cell-type and/or region-specific effects of DNA methylation in establishing sex differences in these cell types.

In Chapter Three, I explore the roles of both DNA methylation and demethylation and find interesting sex differences in epigenetic regulation of the same cell type (ERα) within two hypothalamic nuclei (ventromedial hypothalamus (VMH) and arcuate nucleus (ARC)). As mentioned above, while neonatal inhibition of DNA methylation feminizes ERα labeling of males in the VMH, the same treatment *masculinizes* ERα labeling in females in the neighboring ARC. Furthermore, knocking down the enzymes that remove methyl marks (presumably leading to an accumulation of DNA methylation) results in males having high, female-like levels of ERα. This suggests that methyl marks may be inhibitory (as widely regarded) to ERα gene expression in the VMH, but associated with increased ERα gene activation in the ARC. We probe the methylation status of the ERα gene (*Esr1*) promoter region at weaning and, consistent with our hypothesis, find that male mice have more methylation of the ERα promoter than females in the VMH, while females have more methylation than males in the ARC.

Finally in Chapter Four, we hypothesize that neurochemical expression in the hypothalamus may be largely similar in male and female mice early in life, and become more "specialized" closer to weaning in preparation for sex-specific behaviors (*e.g.*, copulatory or maternal behavior) and physiology. Specifically, we test the prediction that several sub-populations of neurons in the ventromedial hypothalamus of males and females co-express *Esr1*

at birth, but that *Esr1* is subsequently downregulated in males in those sub-populations associated with female-specific behaviors or physiology.

1.2 Introduction to Epigenetics

The word "epigenetics" was coined by Conrad Waddington to explain the observation that myriad phenotypes could arise from the same genotype (Waddington, 1956). The definition was later modified to include the *heritable* nature of the epigenome during cell division in developmental biology (Holliday & Pugh, 1975). However, this definition excludes the role of epigenetics in non-dividing cells, such as neurons, where epigenetic modifications to chromatin have been shown to be important for many processes. Here, we define epigenetics as long-lasting covalent modifications to chromatin that alter gene expression, without reference to heritability.

The backbone of DNA is composed of phosphate groups which carry a negative charge. Cells can pack an incredible amount of DNA into a single nucleus (each human cell contains about 6 feet of DNA) by spooling the DNA around positively charged histone proteins. DNA and its associated proteins are termed *chromatin*, and the basic unit of chromatin is the *nucleosome*: ~147 base pairs of DNA wrapped around a "core" of eight histone proteins (Varshavsky & Georgiev, 1975). The N-terminal region of histones contain a long, amino acid tail that can be covalently modified by the addition of methyl, acetyl, lysine, ubiquitin, or phosphate groups, among others (Bannister & Kouzarides, 2011). Depending on the modification, these marks may either repress or activate the expression of neighboring genes.

In addition, DNA itself can be chemically modified, most frequently by the addition of methyl groups on the 5^{th} carbon of cytosine. This generally correlates with gene repression and is catalyzed by DNA methyltransferases (DNMTs; Jeltsch, 2006; Miranda & Jones, 2007). Although it was initially believed that methylation was restricted to cytosines followed by

guanines (CpG, where p indicates the phosphate group linking two nucleotides), it is now appreciated that methylation can occur on cytosines followed by other nucleotides, especially adenine (A). In fact, in contrast to the rest of the body, DNA methylation in the adult brain occurs just as frequently at CpH sites (H indicating A, C, or thymine (T)) as at CpG sites (Lister et al., 2013), and recent work demonstrates that adenine can also be methylated in the mouse brain (Yao et al., 2017). For the most part, the role of non-CpG methylation in sexual differentiation has not yet been explored.

DNA methylation marks can be quite stable, but can also be removed through a series of steps catalyzed by ten-eleven-translocation (TET) enzymes which begins with the oxidation of methylcytosine (mC) to hydroxymethylcytosine (hmC) (Tahiliani et al., 2009). TETs can further oxidize hmC to formylcytosine, which can then be oxidized to carboxylcytosine. Both products can be converted into an unmodified cytosine through the base excision repair pathway (Gong & Zhu, 2011). Recent work suggests that hmC may be a stable epigenetic mark in its own right, and not just an intermediary in demethylation (Bachman et al., 2014). In support of this suggestion, hmC marks are primarily located in enhancers and gene bodies of actively transcribed genes, whereas mC is frequently found in intergenic repetitive elements (Wen et al., 2014; Wen & Tang, 2014; I.-H. Lin et al., 2017), suggesting that hmC is not simply a step in the process of demethylation. In contrast to mC, hmC marks generally correlate with gene expression (I.-H. Lin et al., 2017), but the role of hmC in sexual differentiation is just beginning to be explored (Cisternas, Cortes, Bruggeman et al., 2020).

As described below, there are sex differences across multiple levels of the epigenetic process (*e.g.*, expression or activity of the enzymes that place or remove epigenetic marks, the global presence of particular epigenetic marks, and in “readers” of epigenetic marks), supporting

the overall hypothesis that epigenetic mechanisms play an important role in sexual differentiation of the brain.

1.3 Gonadal steroid hormones drive sexual differentiation and are epigenetic modifiers

Although sex determination in mammals is genetic, the main role of the X and Y chromosomes in sexual differentiation is to determine what hormones are produced. The Y chromosome contains the *Sry* gene which initiates a cascade of molecular events during embryonic development that leads to the development of testes. Decades of research supports the idea that sexual differentiation in mammals depends on the steroid hormones secreted from the testes (primarily testosterone and its estrogenic metabolites). Gonadal steroids bind to their intracellular receptors, which function as transcription factors to alter gene expression and cause masculinization of the body (M. J. Tsai & O'Malley, 1994; Wu & Tollkuhn, 2017). Circulating gonadal steroids also enter the brain (McEwen et al., 1975) and orchestrate masculinization and defeminization of the nervous system by altering the expression of genes involved in cell death, neurogenesis, synapse formation, and other functions (Lenz & McCarthy, 2010).

It can be argued that many, if not most, actions of steroid hormones involve epigenetic modifications. In classic gonadal steroid hormone action, the steroid binds to its intracellular receptor, which then binds to steroid response elements near the promoter regions of target genes. Co-activators and co-repressors are then recruited that modify the chromatin to increase or (less often) decrease gene expression. For example, steroid receptor coactivator-1 (SRC1) is a histone acetyltransferase (HAT) which primarily acts on histone 3 (Spencer et al., 1997; J. Xu & Li, 2003; Molenda-Figueira et al., 2008), and female rats with genetic ablation of SRC1 are resistant to the defeminizing effects of testosterone on sexual behavior (Auger et al., 2000). These "mini" epigenetic modifications in association with steroid hormone action occur both in

peripheral cells (Yaşar et al., 2016) and in the brain (Tetel et al., 2009). It therefore may not be surprising that an increasing number of studies have offered direct demonstrations that: 1) there are sex differences in the brain epigenome, and 2) hormone-dependent brain sexual differentiation requires epigenetic mechanisms (McCarthy et al., 2009; Forger et al., 2016; Forger, 2018; McCarthy, 2019).

1.4 An extreme sex difference in epigenetic regulation: X chromosome inactivation

X chromosome inactivation is often overlooked when thinking about sex differences in the epigenome but is a process that may have repercussions for epigenetic control of gene expression in all female cells. Because female mammals have two X chromosomes, they have a double dosage of X chromosome genes. However, one X chromosome is randomly inactivated in each XX cell during embryogenesis, presumably to minimize sex differences in the expression of X-linked genes (Lyon, 1961). This process begins with the coating of one X chromosome with *Xist* long-non-coding RNA, followed by recruitment of chromatin silencers (histone deacetylases and methyltransferases), and DNMT3b-directed DNA methylation (Fang et al., 2019).

Thus, the epigenetic silencing of nearly an entire chromosome (but note that a small number of genes escape X-inactivation) occurs in every cell in the body and brain of females, and in no cells of males. Moreover, the inactivation state must be actively maintained throughout the life of the cell (Maxfield Boumil & Lee, 2001). If there were no other compensatory mechanisms, the inactivated X chromosome might create a “heterochromatic sink” that limits the availability of epigenetic machinery that would otherwise act on other chromosomes (Zuckerkandl, 1974); indeed, under carefully controlled experimental conditions, the expression of hundreds of autosomal genes is sensitive to sex chromosome complement (Wijchers et al., 2010). To overcome this, XX cells may require increased expression of DNA methylation

enzymes, enzyme efficiency, or substrate material to meet the higher demand of epigenetic regulation in each and every cell. Either way, it is important to remember that the purpose of the largest known epigenetic sex difference is to make gene expression more equal in males and females. In other words, even when the sexes do not differ with respect to gene expression, the epigenetic underpinnings may be very different.

1.5 Sex differences in the human epigenome

Sexual differentiation in humans occurs mainly during the second trimester, with testosterone levels elevated from about 10 to 20 weeks of gestation in male fetuses (Siiteri & Wilson, 1974). One study examining DNA methylation genome-wide in the human fetal brain found about 8,000 differentially methylated gene loci between the sexes; the vast majority of these were on the sex chromosomes and only 6% were on autosomes (Spiers et al., 2015). The autosomal sex differences in methylation were associated with ~500 genes, with half of the loci hypermethylated and half hypomethylated in females (Spiers et al., 2015). Interestingly, most sex differences in DNA methylation emerged at around 14 weeks post conception, coinciding with peak testosterone levels in male fetuses. This suggests that gonadal hormones may shape sex differences *in utero* in the human brain methylome.

In another study, DNA methylation was examined in the dorsolateral prefrontal cortex of humans from 14 weeks gestation to adulthood. About 5% of CpG sites on autosomes differed between the sexes and the majority of these sites had greater methylation in females (Numata et al., 2012). Similarly, the majority of genetic loci with sex differences in DNA methylation in the frontal cortex of adults showed greater methylation in women (H. Xu et al., 2014). Interestingly, about 20% of the genes that were differentially methylated in the adult frontal cortex had sex

differences in the same direction in fetal cortical tissue, suggesting a subset of genes with stable sex differences across the lifespan (H. Xu et al., 2014; Spiers et al., 2015).

Given the barriers to obtaining large numbers of neural samples, many studies on the human epigenome have relied on peripheral tissues, such as blood cells. Although one should proceed with caution when using peripheral samples as a surrogate for the brain, there is some support for this approach based on correlations in DNA methylation between blood cells and brain samples (Davies et al., 2012; Horvath et al., 2012). At birth, umbilical cord blood cells contain a small number of sex differences in methylation of CpG sites on autosomes, and most of these sites have higher methylation in girls (Adkins et al., 2011; Yousefi et al., 2015). A bias towards greater DNA methylation in females is also found when examining differentially methylated regions (as opposed to single CpG sites; Yousefi et al., 2015). Many of the differentially methylated marks were found near genes associated with nervous system development, suggesting that – if the same pattern is seen in the brain – DNA methylation contributes sex-specifically to brain development. Saliva samples from adults reveal a higher number of sexually differentiated CpG sites on autosomes, relative to fetal brain and newborn blood— and similarly most of these have increased methylation in females, even when accounting for sex differences in cellular composition (J. Liu et al., 2010; Sun et al., 2014; Inoshita et al., 2015; Zaghlool et al., 2015).

Thus, work to date illustrates that DNA methylation in the human brain differs between the sexes starting before birth and suggests a sex bias in DNA methylation, such that more differentially methylated genetic loci are hypermethylated in females than in males. A limitation of these studies (and several of the rodent studies mentioned below) is that the methods used do not distinguish between methylation and hydroxymethylation. This is important, given that mC

marks are associated with gene repression, while hmC marks are associated with gene activation. Interestingly, in lymphocytes from umbilical cord blood of newborns, girls had twice the number of "methylated" CpG sites within CpG islands (regions with a high density of CpG sites, typically located near promoters), transcriptional start sites, and shores/shelves (regions located up to 4kb from CpG islands; Maschietto et al., 2017). Boys, on the other hand, had more "methylated" CpG sites in gene bodies and within the 3' untranslated region. Given that these different gene locations are associated with mC and hmC, respectively, it is important for future experiments to use techniques that distinguish between these marks to investigate their independent role in shaping the male and female brain.

1.6 Sex differences in the brain epigenome: rodent studies

Animal studies allow researchers to investigate sex differences in the neural epigenome in tightly controlled settings. This is especially important given that many epigenetic studies have demonstrated that the environment substantially alters the epigenome. By eliminating group differences in diet, stress, etc., these confounding variables can be ruled out, and the effects of sex on the epigenome can be studied in isolation. Rodent studies also allow for the manipulation of epigenetic processes during critical periods of development, which is not possible in human studies.

The preoptic area (POA) of the hypothalamus has been the target of intense scrutiny as a brain region where many morphological and neurochemical sex differences have been described in rodents. Most of these sex differences are due to differential exposure to perinatal testosterone, or an estrogenic metabolite of testosterone (such as estradiol), in males and females (McCarthy et al., 2009). The first study to report sex differences in an epigenetic mark in the rodent brain used pyrosequencing to examine DNA methylation patterns associated with steroid hormone

receptor genes in the POA of rats. The authors reported sex differences in the intron 1 promoter region of the gene for estrogen receptor alpha (*Esr1*; Schwarz et al., 2010). Importantly, the difference(s) were reversed by treating newborn females with estradiol, indicating that they are due to perinatal gonadal steroid exposure. In addition, sex differences in the epigenome were dynamic across the lifespan, as sex differences in CpG sites disappeared and reappeared at different locations with age (Schwarz et al., 2010). Similarly, male rats have increased CpG methylation within the *Esr1* 1b promoter in the POA on postnatal day (P) 8, and treating female pups with estradiol on the first two days of life masculinizes (increases) CpG methylation relative to control-treated females (Kurian et al., 2010).

A later study found that female rats have higher global levels of DNA methylation in the POA on the first days of life than do males (Nugent et al., 2015). Again, perinatal gonadal steroids are the cause of this difference because it is eliminated when females are treated with estradiol at birth (Nugent et al., 2015). We recently reported that female mice also have higher global levels of DNA methylation in the POA during neonatal life, suggesting that this epigenetic sex difference is conserved across rodent species (Cisternas, Cortes, Bruggeman et al., 2020). The Nugent et al. (2015) study also showed that many sex differences in DNA methylation function to converge gene expression. For example, while inhibition of DNA methylation early in life abolishes some sex differences in gene expression in the rat POA, it causes many more genes to become differentially expressed in males and females (Nugent et al., 2015). This surprising result implies that sex differences in DNA methylation may *primarily* function to equalize gene expression between male and female brains. If so, then any global perturbation to DNA methylation might uncover a plethora of sex differences in neural circuit functioning where they did not previously exist.

Ghahramani and colleagues examined the effects of neonatal testosterone treatment on DNA methylation throughout the genome in the POA and neighboring BNST (POA/BNST) as well as the striatum of male and female mice. Female mice treated with testosterone at birth had DNA methylation patterns that were significantly more masculine than control females, although not fully masculinized (Ghahramani et al., 2014). Interestingly, the large majority of sex differences and effects of neonatal testosterone on the methylome were not observed perinatally, but only emerged in adulthood (Ghahramani et al., 2014). This was seen despite the fact that all mice in the study were gonadectomized prior to puberty (*i.e.*, effects could not be attributed to sex differences in adult levels of circulating gonadal steroids). Thus, perinatal exposure to gonadal steroids leads to widespread, but latent differences in the epigenome that emerge later in life, in what may be termed an "epigenetic echo" (McCarthy et al., 2017).

1.7 Sex differences in epigenetic writers, erasers, and readers

The previous section demonstrates that many sex differences in epigenetic marks in the brains of rodents are due to exposure to gonadal steroids early in life. The question then becomes: how do steroid hormones cause those changes? One important mechanism may be via hormonal regulation of the expression of epigenetic "writers" and "erasers," the enzymes that, respectively, place and remove epigenetic marks, or by altering the "readers" that interpret those marks.

Three DNMTs, the "writers" of DNA methylation, are expressed in the brain (*Dnmt1, Dnmt3a, Dnmt3b*). In addition, the brain expresses *Dnmt3L,* which does not itself have enzymatic activity but increases the efficiency of DNMT3a and DNMT3b (Hata et al., 2002; Jia et al., 2007). We recently found that female mice have greater expression of *Dnmt1* and *Dnmt3L* than males within the neonatal POA (Cisternas, Cortes, Bruggeman et al., 2020) (Figure 1-1).

Females administered testosterone on the first two days of life have intermediate expression, not significantly different from control males or females, demonstrating at least partial regulation by postnatal gonadal hormones. In neonatal rats, no sex difference in DNMT expression in the POA was found, but females had greater DNMT *catalytic activity* during the first few days of life than did males (Nugent et al., 2015). In this case, estradiol treatment completely masculinized DNMT activity. Future studies will need to test whether sex differences in DNMT3a (or DNMT3b) activity depend on DNMT3L and whether DNMT3L is required for establishing sex differences in the brain.

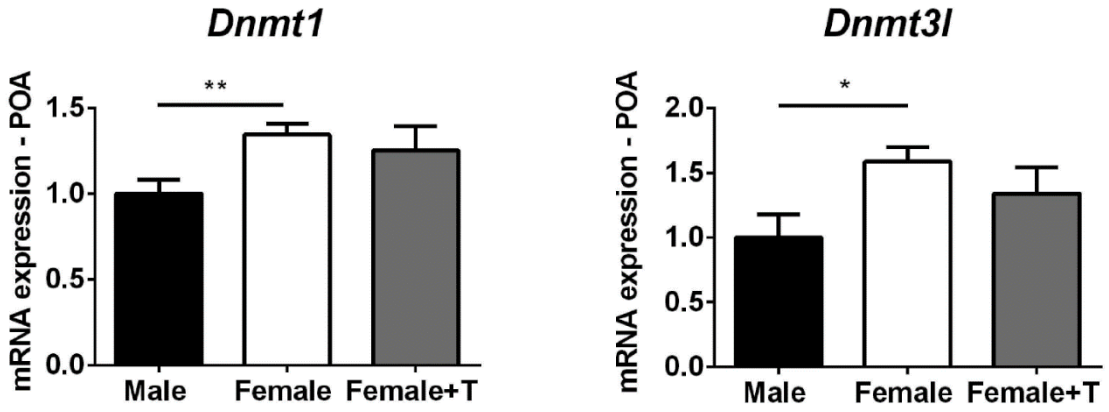

Figure 1-1 Female mice have higher expression of Dnmt1 and Dnmt3L in the preoptic area of the hypothalamus (POA) at postnatal day (P) 7*. Dnmt1 expression in females treated with testosterone was intermediate and not significantly different from that of control males or females. *P <0.05. ** P < 0.01. Data are mean ± SEM. N = 5 vehicle females, N = 7 males and females + testosterone. From Cisternas, Cortes, Bruggeman et al., 2020.*

Sex differences in DNMT activity or expression presumably contribute to hypermethylation in the female POA after birth (Nugent et al., 2015). However, total DNA methylation depends on the balance between the placement and removal of methyl marks, so sex differences in DNA methylation could also be due to demethylation by the TET enzymes (the DNA methylation "erasers"). Indeed, we found that, compared to females, male mice have higher *Tet1* and *Tet2* expression in the VMH on P1, and higher *Tet2* and *Tet3* expression in the

POA on P7 (Cisternas, Cortes, Bruggeman et al., 2020) (Figure 1-2). Treatment of females with testosterone at birth partially masculinized expression of *Tet2* and *Tet3* (Cisternas, Cortes, Bruggeman et al., 2020). Similarly, in cancer cell lines, estradiol upregulates *Tet2* within 24 hours and leads to demethylation of estrogen-regulated genes (Wang et al., 2018).

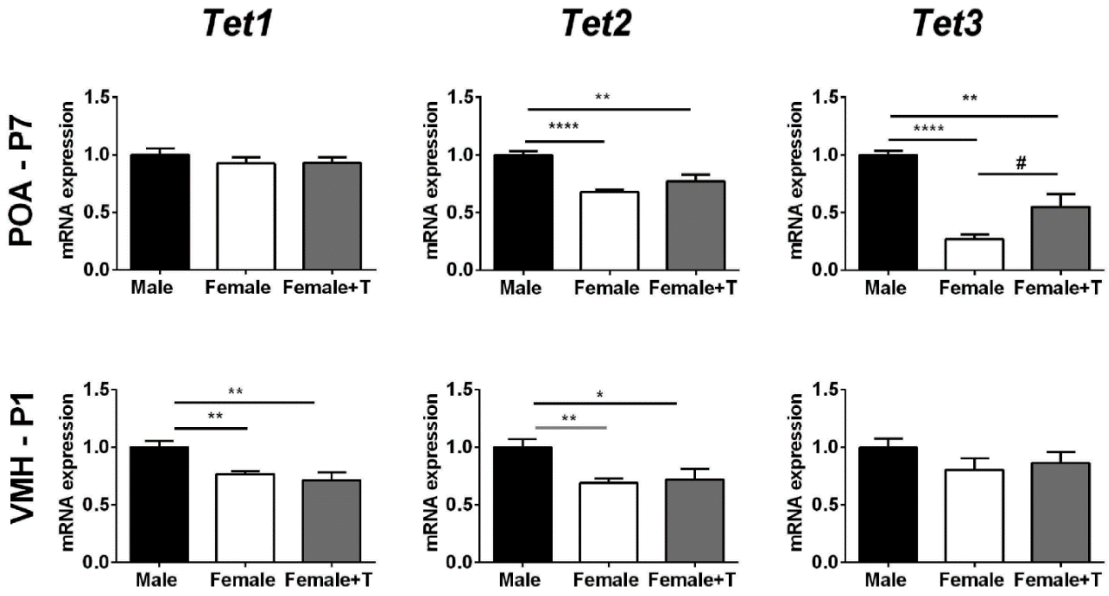

Figure 1-2 Sex differences in TET expression are observed during neonatal life*. In the preoptic area of the hypothalamus, Tet2 and Tet3 expression are higher in males than in females on postnatal day (P) 7. Neonatal testosterone treatment partially masculinized Tet3 expression in females, but did not affect the expression of Tet2. N = 5-7 per group. In the ventromedial nucleus of the hypothalamus, expression of Tet1 and Tet2 is higher in males on P1, and neonatal testosterone treatment of females did not eliminate the sex difference. N = 6 in each female group, N = 13 males. # P < 0.1, *P < 0.05, **P < 01, ****P < 0.0001. Data are mean ± SEM. From Cisternas, Cortes, Bruggeman et al., 2020.*

Epigenetic "readers," such as the methyl-CpG-binding domain (MBD) proteins, are recruited to epigenetically modified chromatin to regulate transcription (Nan et al., 1998; Ballestar & Wolffe, 2001). Newborn female rats have higher levels of the MBD family member methyl-CpG-binding protein 2 (MeCP2) in the amygdala and VMH of the hypothalamus (Kurian

et al., 2007). Mutations in the MeCP2 gene in humans cause Rett syndrome, a severe neurodevelopmental disorder, illustrating the importance of "reading" the epigenetic code.

1.8 Manipulating epigenetic marks during development: effects on sexual differentiation of neural morphology and behavior

The studies described so far do not distinguish whether any particular epigenetic mechanisms are *required* for brain sexual differentiation. To address that question, it is necessary to disrupt an epigenetic process during the period of sexual differentiation (the first week or so of life in rodents) and then determine whether sex differences in the brain and behavior are disrupted later in life.

The first study to manipulate epigenetic marks to test their role in sexual differentiation of the brain administered a histone deacetylase (HDAC) inhibitor and discovered that masculinization of neuronal number in the principal nucleus of the BNST (BNSTp) was blocked (Murray et al., 2009). A following study administered an HDAC inhibitor into the cerebral ventricles of newborn rats and found that male sexual behavior was disrupted in adulthood (Matsuda et al., 2011). In a landmark study of similar design, Nugent et al. (2015) gave intracerebroventricular injections of the DNA methyltransferase inhibitor zebularine to newborn rats on the first two days of life. The inhibition of DNA methylation masculinized dendritic spine density of neurons in the POA of females and sexual behavior in adulthood (Nugent et al., 2015). Interestingly, zebularine treatment or genetic downregulation of DNMTs outside of the critical period for sexual differentiation (which is generally thought to end after postnatal day 10) also partially masculinized the copulatory behavior of females, suggesting an extended period during which epigenetic programming may affect brain differentiation, and that may depend on the availability of epigenetic factors.

There is a sex difference in MeCP2 expression on P1 in the rat amygdala, a brain region that has been implicated in the control of social play behavior (Kurian et al., 2007). When Kurian and colleagues used small interfering (si)RNAs to disrupt MeCP2 expression during the first three days of life, sex differences in juvenile social play were abolished (Kurian et al., 2008). In addition, one type of MeCP2 mutation seen in Rett syndrome inhibits the ability of MeCP2 to bind to nuclear receptor co-repressor (NCoR; Lyst et al., 2013). NCoR binds to Kaiso and other MBD proteins and represses neural gene expression by recruiting HDACs (Ordentlich et al., 1999; Yoon et al., 2003). Interestingly, there are also sex differences in NCoR expression in areas of the brain with high sex steroid receptor expression. Male and estradiol-treated females have lower levels of NCoR mRNA in the developing hypothalamus and amygdala than control females (Jessen et al., 2010). Moreover, a transient reduction of NCoR expression in the neonatal amygdala disrupts sex differences in juvenile play and anxiety-like behavior, by feminizing the behavior of males (Jessen et al., 2010).

Thus, coordinated changes in epigenetic marks or the readers of those marks, often orchestrated by testosterone or its metabolites, are required for sexual differentiation of brain anatomy and behavior.

1.9 Manipulating epigenetic marks during development: sexual differentiation of neurochemistry

The sex differences that received the most attention early on were structural, for example, differences in brain area volume, neuron number, or dendritic morphology. Recently, sequencing across 44 human tissues suggests that sex differences in gene expression are ubiquitous, although often small in magnitude (Oliva et al., 2020). The most common, and most understudied, type of sex difference in the brain is likely to be sex differences in neurochemical phenotype. The

chemical phenotype of a cell is defined based on its stable gene expression patterns. As mentioned above, for example, female rodents have more neurons expressing kisspeptin and tyrosine hydroxylase in the AVPV (Semaan & Kauffman, 2010), while males have a greater number of vasopressin-expressing neurons in the BNSTp (de Vries et al., 2008). These sex differences are determined by gonadal steroid exposure early in life (de Vries & Panzica, 2006; Brock & Bakker, 2013) and regulate important physiological and behavioral processes, such as puberty (Terasawa et al., 2013) and social behavior (Koolhaas et al., 1990; Veenema et al., 2013). Females also have more neurons expressing ERα in the VMH and ARC of the hypothalamus, and these cells sex-specifically control energy and bone homeostasis (Y. Xu et al., 2011; Correa et al., 2015; Herber et al., 2019; van Veen et al., 2020).

Cellular diversity depends on the carefully orchestrated expression of specific transcription factors during development, and culminates in unique epigenomic signatures across different tissue types, brain regions, and cell types within the same region (Ladd-Acosta et al., 2007; Ghosh et al., 2010; Mo et al., 2015). Throughout the body, DNA methylation controls cellular differentiation by silencing cell fate regulators (Reik et al., 2003) and plays a significant role in maintaining cell phenotype throughout life. Therefore, we hypothesize that sex differences in neuronal phenotype are the result of differences in epigenetic mechanisms, including DNA methylation.

We first tested this by administering a DNA methylation inhibitor, zebularine, at birth directly into the brains of male and female mice. Early life inhibition of DNMT activity had a lasting effect on the number of cells expressing ERα in the VMH and the POA (Mosley, Weathington et al., 2017). Females normally have more ERα cells in both regions and neonatal zebularine treatment increased ERα cell number in males, thereby reducing or eliminating the

sex differences. Conversely, males have more cells that express calbindin in the POA, and zebularine treatment also affected this sex difference, by increasing calbindin cell number in females (Mosley, Weathington et al., 2017). Therefore, inhibition of DNA methylation can cause both masculinization and feminization of neuronal phenotype, depending on the brain region or cell type. I contributed to this study by showing that zebularine did not alter cell death markers or the total number of surviving cells at weaning, demonstrating that DNA methylation determines the proportion of cells expressing these markers, rather than the number of remaining cells (Mosley, Weathington et al., 2017).

Males have greater expression of the neuropeptide vasopressin in the amygdala than do females, and this sex difference also depends on DNA methylation. siRNA injections against MeCP2 during the first three days of life abolish sex differences in vasopressin expression in the amygdala of rats two weeks after birth by feminizing males (Forbes-Lorman et al., 2012). Androgen receptor and galanin are similarly decreased to female levels, while ERα is unaffected. By adulthood, vasopressin fibers remain feminized in the amygdala, lateral septum, and BNST in males treated with siRNA, but androgen receptor and galanin return to baseline.

Taken together, DNA methylation early in life may be critical for establishing sex differences in the brain. Thus, in this dissertation, I test the hypothesis that epigenetic mechanisms, such as DNA methylation, play an important role in the development of the most common type of sex difference in the brain, neurochemical phenotype. In Chapter 2, we investigate the interplay between gonadal hormones and DNA methylation and determine their effects on several sex differences in neurochemistry in the mouse hypothalamus. In Chapter 3, I focus on the roles that DNA methylation and TET-mediated demethylation have on sex differences in one neurochemical phenotype, ERα expressing cells, in two neighboring

hypothalamic regions. Finally, in Chapter 4, I follow up on the observation from earlier chapters that male and female mice are born with equal numbers of neurons expressing ERα, but males downregulate ERα in about half of those neurons by weaning. I therefore characterize developmental changes in *Esr1*, and other cell markers co-expressed with *Esr1*, to test the hypothesis that specific sub-populations of ERα cells may be preferentially reduced in males during development.

2 NEONATAL INHIBITION OF DNA METHYLATION DISRUPTS TESTOSTERONE-DEPENDENT MASCULINIZATION OF NEUROCHEMICAL PHENOTYPE

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[1]These authors contributed equally to this work.

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Contribution to publication: CD Cisternas and I performed mouse injections and collections. I performed the histological processing and analysis for ERα and kisspeptin.

2.1 Introduction

Many sex differences in the mammalian brain are established by a transient, perinatal exposure to gonadal testosterone in males (Morris et al., 2008; Lenz et al., 2012; Forger et al., 2016). In some cases, testosterone regulates neuronal cell death to cause sex differences in neuron number (Forger, 2006, 2009b); however, other sex differences persist even if developmental cell death is eliminated. For example, males have more neurons expressing calbindin in the medial preoptic area of the hypothalamus (mPOA; Büdefeld et al., 2008;

Edelmann et al., 2007) and vasopressin in the bed nucleus of the stria terminalis (BNST; De Vries et al., 1984; Rood et al., 2013), whereas females have more neurons expressing tyrosine hydroxylase and kisspeptin in the anteroventral periventricular nucleus and neighboring rostral periventricular nucleus (AVPV/PeN; Simerly et al., 1997; Clarkson & Herbison, 2006; Kauffman et al., 2007). These sex differences all persist in mice lacking the pro-death gene, *Bax* (Forger et al., 2004; de Vries et al., 2008; Semaan et al., 2010; Gilmore et al., 2012), despite the near complete elimination of developmental neuronal cell death in *Bax* knockout mice (White et al., 1998; Ahern et al., 2013).

Epigenetic modifications to chromatin control gene expression and are required for the differentiation of cell phenotype throughout development. Two of the best studied epigenetic modifications are the acetylation of histone tails and the methylation of cytosine residues of DNA, and both have been implicated in the sexual differentiation of brain anatomy and behavior (Murray et al., 2009; Matsuda et al., 2011; Nugent et al., 2015). DNA methylation is controlled by a family of DNA methyltransferases (DNMTs) that place methyl marks, and ten-eleven translocases (TET enzymes) that remove those marks (Klose & Bird, 2006; Ooi et al., 2009; Tahiliani et al., 2009). DNA methylation is normally associated with gene repression, although there are exceptions (Goll & Bestor, 2005). The expression of DNMT enzymes peaks during the first postnatal week in the mouse brain (Cisternas, Cortes, Bruggeman, et al., 2020; Lister et al., 2013), which coincides with the critical period for testosterone-dependent sexual differentiation (Figure 2-1). Moreover, there are sex differences in DNMT and TET activity and/or expression in the neonatal brain (Cisternas, Cortes, Bruggeman, et al., 2020; Nugent et al., 2015). We therefore hypothesized that sex differences in neurochemical phenotype (*i.e.*, the number of cells expressing specific markers) may depend on differential DNA methylation in males and females.

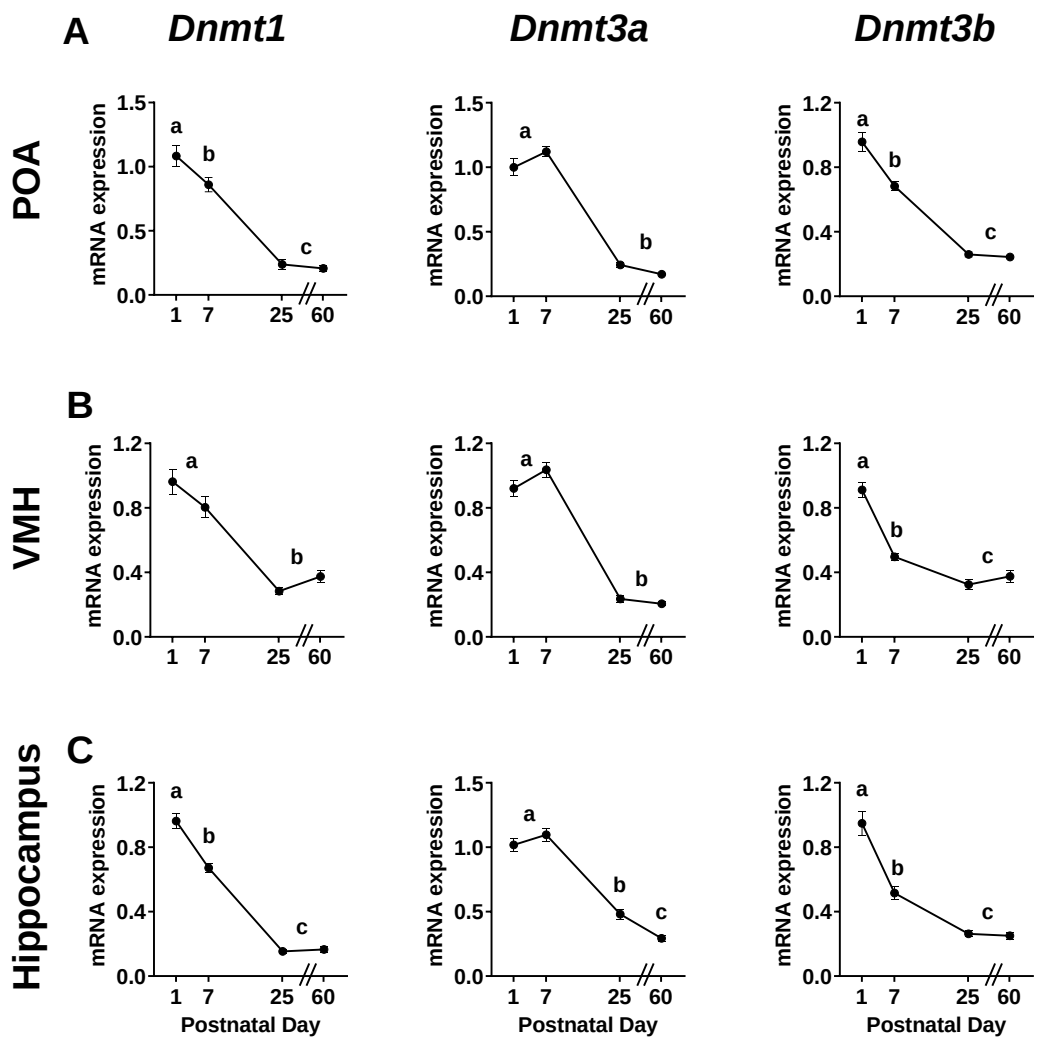

Figure 2-1 DNMT expression highest during neonatal life. *Dnmt1, Dnmt3a, and Dnmt3b mRNA expression at postnatal days 1, 7, 25, and 60 in (A) the preoptic area of the hypothalamus; (B) the ventromedial nucleus of the hypothalamus; and (C) the hippocampus. The expression of Dnmt1 and Dnmt3b was highest on P1, then dropped to lower levels by P7, whereas expression of Dnmt3a was remained elevated throughout the first postnatal week in all brain regions Different letters indicate significant differences. Data are mean ± SEM. N = 19-20 mice per age, sexes combined. From Cisternas, Cortes, Bruggeman et al., 2020.*

In a first test of this idea (Mosley, Weathington, et al., 2017), we administered a DNMT inhibitor to newborn male and female mice, and examined effects on the male-biased sex difference in calbindin cell number in the mPOA, and the female-biased sex difference in the number of estrogen receptor (ER) α cells in the ventrolateral portion of the ventromedial hypothalamus (VMHvl; Kühnemann et al., 1994; Yokosuka et al., 1997; Cao & Patisaul, 2011). The neonatal inhibition of DNA methylation increased the number of cells expressing both cell types at weaning (Mosley, Weathington, et al., 2017), consistent with the canonical association of DNA methylation with the suppression of gene transcription, and also reduced or eliminated the sex differences in calbindin and ERα cell number (Mosley, Weathington, et al., 2017).

Calbindin cell number in the mPOA is masculinized in female rats and mice treated with testosterone or estradiol at birth, and the sex difference is present prior to puberty (Gilmore et al., 2012; Orikasa & Sakuma, 2010; Sickel & McCarthy, 2000). The sex difference in ERα in the VMHvl is also evident prior to puberty in rats and mice (Yokosuka et al., 1997; Brock, De Mees, et al., 2015; Mosley, Weathington, et al., 2017), although its dependence on neonatal testosterone has not yet been demonstrated. Here, we hypothesized that testosterone causes these sex differences in cell phenotype (a decrease in ERα and an increase in calbindin) by orchestrating changes in DNA methylation around the time of birth. If so, then effects of endogenous or exogenous testosterone may be prevented by inhibiting DNA methylation.

To test this, we administered a masculinizing dose of testosterone to female mice concomitant with intracerebroventricular (ICV) injections of a DNMT inhibitor or vehicle during the critical period of sexual differentiation, and examined effects on calbindin in the mPOA and ERα in the VMHvl at weaning. We also extended our observations to two additional sex differences in neurochemical phenotype: calbindin cell number in the BNST (which is normally

greater in males; Gilmore et al., 2012) and kisspeptin cell number in the AVPV/PeN (greater in females; Clarkson & Herbison, 2006; Semaan et al., 2010). We find that neonatal inhibition of DNA methylation eliminates or reduces sex differences in calbindin and ERα. Interestingly, it does so by increasing cell number specifically in those groups in which the cell type of interest is normally repressed (*i.e.*, calbindin cells in females and ERα cells in males and testosterone-treated females).

2.2 Methods

2.2.1 Animals

Wildtype C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, ME). Breeding pairs were housed in a 12:12 light:dark cycle at 22° C with food and water available *ad libitum* and were checked daily for births. All procedures were performed in accordance with the National Institutes of Health animal welfare guidelines and were approved by the Georgia State University Institutional Animal Care and Use Committee.

2.2.2 Zebularine injections

DNA methylation was inhibited using zebularine (Calbiochem, San Diego, CA), a global DNMT inhibitor that has been used in many rodent studies due to its low toxicity (Anier et al., 2010; Dock et al., 2015). Cryoanesthetized pups received ICV injections of 300 ng zebularine into each hemisphere (in 500 nL 10% dimethyl sulfoxide, 90% physiological saline), or the vehicle alone, on postnatal day (P) 0 (the day of birth) and P1, as previously (Mosley, Weathington, et al., 2017). A 30-gauge needle attached to a 5µl Hamilton syringe was lowered 2 mm below the skull, at approximately 1 mm rostral to lambda and 1 mm lateral to the sagittal suture. Zebularine or vehicle was injected at a rate of 33 nL/sec using a Micro4 microsyringe pump (World Precision Instruments, Sarasota, FL).

2.2.3 Testosterone injections and brain collection

Concomitant with zebularine or vehicle injections, female newborns received subcutaneous injections of either testosterone propionate (Sigma, St Louis, MO; 100 μg in 25 μL of peanut oil) or the oil vehicle on P0 and P1; all males received the vehicle only. Animals were sacrificed at weaning on P25, as previously (Mosley, Weathington, et al., 2017), to avoid effects of pubertal hormones. Brains were fixed by immersion in 5% acrolein for 24 hours, then transferred to 30% sucrose in 0.1 M phosphate buffer before sectioning into four coronal series of 30 microns. Sections were stored in cryoprotectant (30% sucrose, 30% ethylene glycol in 0.1 M phosphate buffer, 1% polyvinylpyrrolidone) until staining.

2.2.4 Immunohistochemistry for calbindin, ERα, and kisspeptin

One series of tissue was stained for calbindin (mouse anti-calbindin, 1:20,000 anti-calbindin-D28k; Sigma; RRID: AB_476894), one for ERα (rabbit anti-ERα, 1:20,000; EMD Millipore, Billerica, MA; RRID: AB_310305), and one for kisspeptin (rabbit anti-kisspeptin, 1:2,000; EMD Millipore; RRID: AB_9754). Protocols are described in detail elsewhere (Mosley, Weathington, et al., 2017). Briefly, on the first day tissue was incubated in 0.1 M glycine for 30 minutes, extensively rinsed in 1X tris (hydroxymethyl)aminomethane-buffered saline (TBS), incubated in a blocking solution (1X TBS, 10% normal goat serum, 1% hydrogen peroxide, and 0.4% Triton-X), followed by an overnight incubation in primary antibody. On the next day, secondary antibodies used were biotinylated goat anti-mouse (1:500 for calbindin, Vector Laboratories, Burlingame, CA), or biotinylated goat anti-rabbit (1:250 for ERα and 1:500 for kisspeptin, Vector Laboratories). Staining was visualized using an avidin-biotin complex followed by incubation in diaminobenzidine-nickel (Vector Laboratories).

2.2.5 Cell-type quantification

The number of cells positive for calbindin in the mPOA and BNST, ERα in the VMHvl, and kisspeptin in the AVPV/PeN was counted with the aid of Stereo Investigator software (MBF Bioscience, Williston, VT). The counting strategy for each cell group was based on the size and cell number of each region, and all analyses were performed by an experimenter blind to the group membership. For calbindin in the mPOA, an ellipsoidal contour (300 μm major axis, 180 μm minor axis) was superimposed around the region of interest. Labeled cells within the contours were counted in the left and right hemispheres and the two highest counts were summed, as previously (Gilmore et al., 2012). Calbindin-positive cells in the encapsulated portion of the BNST were quantified as previously (Kelly et al., 2013) using the particle counter function of ImageJ (Version 1.47; National Institutes of Health, Bethesda, MD). For the VMHvl, a contour was manually drawn on each hemisphere, labeled cells within the contours were counted, and sections with the four highest counts of ERα cells were summed for each animal. For kisspeptin, the AVPV/PeN region was determined using the anterior commissure and third ventricle as landmarks, and all labeled cells in all sections were counted.

2.2.6 Efficacy of zebularine treatment

To confirm the efficacy of our treatments, we examined DNMT activity in a separate cohort of newborns (all males) that received zebularine or vehicle as above, and were sacrificed six or 24 hours after the last injection (P1). The mediobasal hypothalamus was manually dissected and kept at – 80 °C until processing. Nuclear protein was purified using the EpiQuik Nuclear Extraction Kit 1 (Epigentek, Farmingdale, NY; OP-0002) and quantified by BCA Protein Assay (Thermo Scientific; 23252). Total DNMT activity was evaluated using the EpiQuik DNMT Activity Assay Ultra Kit (Epigentek; P-3010), according to the manufacturer

instructions. The DNMT activity was calculated using the formula: DNMT Activity (RFU/h/mg protein) = [(Sample RFU – Blank RFU) / (Protein Amount (μg)* x 2 hours)] x 1000 where RFU are the relative fluorescent units measured.

2.2.7 Statistical analyses

DNMT activity after zebularine injections and *a priori* predictions about sex differences and the effect of neonatal testosterone were evaluated by two-tailed independent t-tests. The effects of group (males, females, masculinized females) and treatment (zebularine, vehicle) on the number of cells expressing specific phenotypes were analyzed with two-way ANOVA using Graph Pad Prism. ANOVA was followed by Fisher's least significance difference (LSD) post hoc test when appropriate.

2.3 Results

2.3.1 Zebularine transiently decreases DNMT activity

Zebularine reduces DNA methylation within one hour in hippocampal slice cultures (Levenson et al., 2006), and ICV injections to adult rats reduce DNA methylation in the brain within four hours (Matt et al., 2018). However, few studies have performed a time course for zebularine effects and, to our knowledge, no studies have examined this in the neonatal brain. To confirm the efficacy of our zebularine injections, DNMT activity was examined in the hypothalamus six or 24 hours after injections of zebularine to newborns on P0 and P1. Compared to vehicle controls, zebularine-treated animals experienced a 54% reduction in global DNMT activity six hours after treatment ($t_6 = 3.32$; $P < 0.02$), and activity had returned to control levels by 24 hours after the last injection ($t_6 = 1.84$; $P > 0.80$, Figure 2-2). Thus, zebularine transiently decreased global DNMT activity.

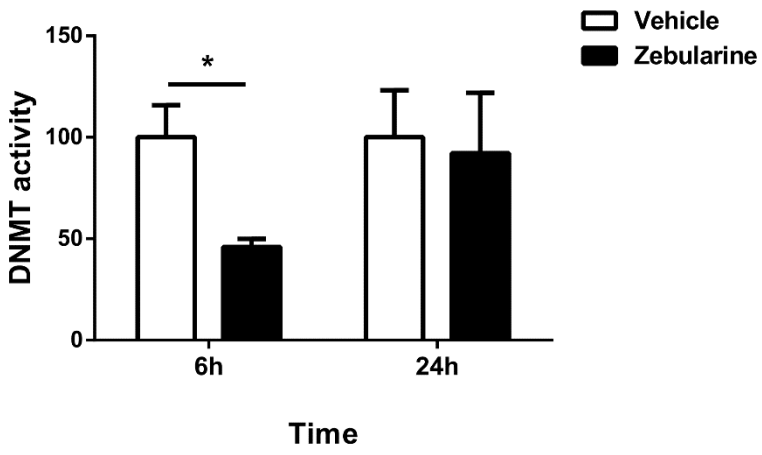

Figure 2-2 DNMT activity is transiently reduced after zebularine treatment. *Compared to vehicle-treated controls, total DNMT activity in the mediobasal hypothalamus was reduced by 54% six hours after ICV zebularine injections in neonatal mice. There was no difference in DNMT activity relative to vehicle controls at 24 hours after treatment. * $P < 0.05$. Data are mean ± SEM. N = 4 for all groups.*

2.3.2 Neonatal inhibition of DNA methylation increases calbindin cell number only in females

As expected, control males had more calbindin-positive cells in the mPOA than control females at weaning ($t_{20} = 3.60$; $P < 0.002$; Figure 2-2). Neonatal testosterone treatment of females increased calbindin cell number ($t_{16} = 5.33$; $P < 0.0001$) and eliminated this sex difference. If the sex difference in calbindin cell number was due to differential DNA methylation among groups, then it might be inhibited by neonatal treatment with zebularine. Indeed, we found a main effect of group ($F_{2, 62} = 10.91$, $P < 0.0001$) as well as a group-by-treatment interaction ($F_{2, 62} = 5.05$, $P < 0.01$) on calbindin cell number in the two-way ANOVA (Figure 2-3). Calbindin cell number was significantly higher in control males and testosterone-treated females than in control females ($P < 0.0001$ for both comparisons). Neonatal zebularine treatment increased calbindin cell number at weaning only in control females ($P < 0.02$) and was

as effective as testosterone in this regard (female + testosterone vs female + zebularine, $P = 0.66$). As a result, group differences were abolished in zebularine-treated mice.

The same general pattern was seen for calbindin cells in the BNST. We confirmed that the sex difference in calbindin cell number previously seen in the BNST of *adults* (Gilmore et al., 2012) is present prior to puberty (control male versus control female, $t_{21} = 2.23$; $P < 0.04$; Figure 2-4). There was a trend for a higher number of calbindin-positive cells in the female + testosterone group compared to control females, but this did not reach significance ($P < 0.1$). By two-way ANOVA, we found a significant effect of zebularine treatment on calbindin cell number ($F_{1, 61} = 4.02$, $P < 0.05$; Figure 2.3): zebularine increased the number of calbindin-positive neurons overall, and within groups this was significant only for females ($P < 0.05$).

These findings suggest that DNA methylation normally decreases calbindin cell number in the mPOA and BNST of females.

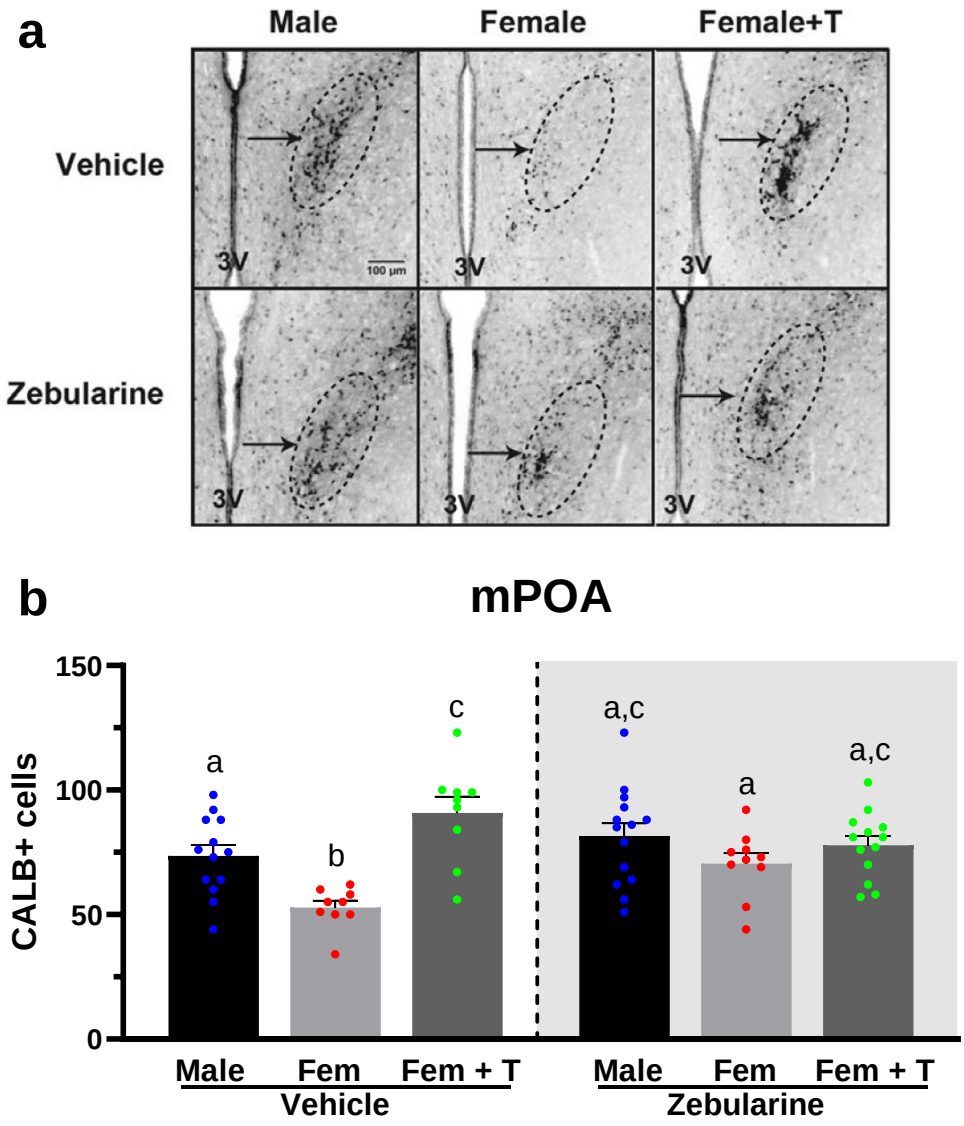

Figure 2-3 Neonatal zebularine increased calbindin cell number in the medial preoptic area (mPOA) only in females. *(A) Photomicrographs showing calbindin-positive (CALB+) cells in the mPOA at weaning in males, females, and testosterone- (T-) treated females that received ICV vehicle or zebularine at birth. 3V: third ventricle. (B) Quantification of CALB+ cell number shows that males and testosterone-treated females had more CALB+ cells on P25 than did control females ($P < 0.01$). Neonatal treatment with zebularine increased CALB+ cell number only in females ($P < 0.05$) and eliminated group differences. Data are mean ± SEM. N = 9-14 per group. If bars share a letter, they do not significantly differ from each other.*

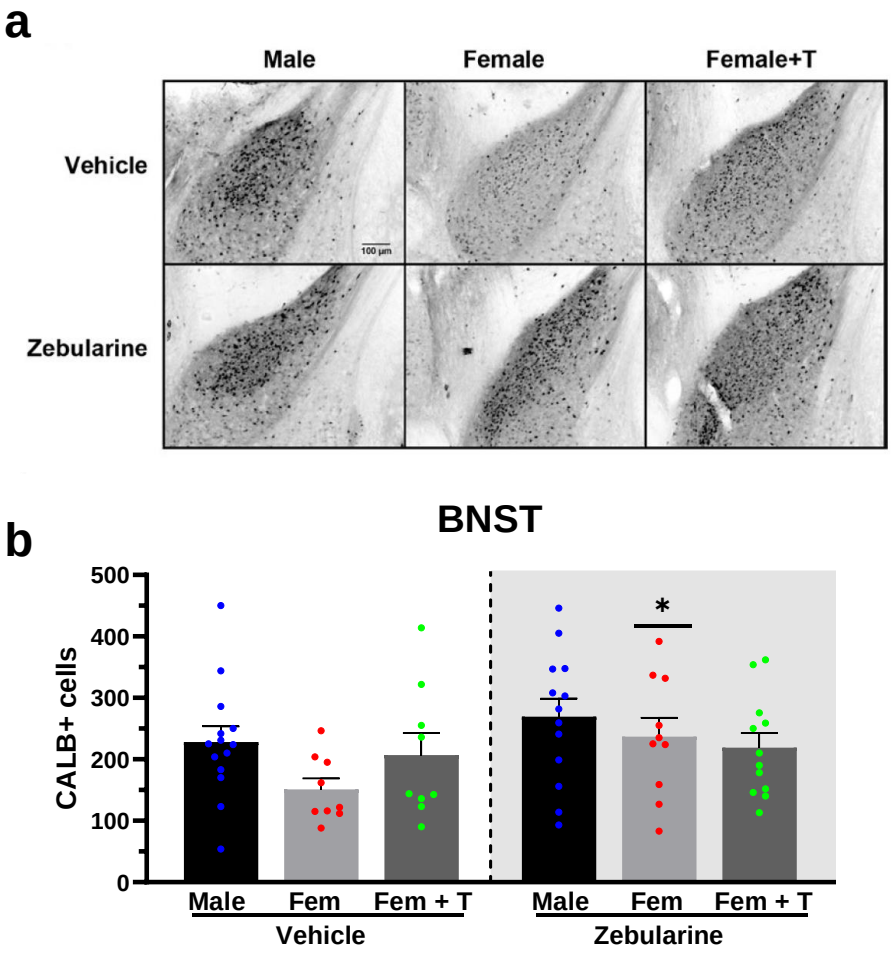

Figure 2-4 Neonatal zebularine increased calbindin cell number in the bed nucleus of the stria terminalis (BNST) only in females. *(A) Photomicrographs showing calbindin-positive (CALB+) cells in the encapsulated portion of the BNST at weaning in males, females, and testosterone- (T-) treated females that received ICV vehicle or zebularine at birth. (B) Quantification of CALB+ cell number at weaning. Control males had more CALB+ cells than control females in an a priori t-test ($P < 0.05$), although the main effect of group in the ANOVA did not reach significance. Neonatal zebularine treatment increased CALB+ cell number overall at P25, and this was significant only for females (* $P < 0.05$). N = 9-14 per group. Data are mean ± SEM.*

2.3.3 Neonatal inhibition of DNA methylation partially prevents the masculinizing effect of testosterone on ERα cell number

In contrast to the male-biased sex differences in calbindin cell number, females have more ERα neurons in the VMHvl than do males. We confirmed this sex difference and found that neonatal testosterone decreased ERα cell number at weaning in females (control female vs testosterone-treated female, $t_9 = 9.16$; $P < 0.0001$) to a level indistinguishable from that in males (Figure 2.5). In the ANOVA, we found significant main effects of group ($F_{2, 35} = 98.58$, $P < 0.0001$) and zebularine treatment ($F_{1, 35} = 4.15$, $P < 0.05$), as well as a group-by-treatment interaction ($F_{2, 35} = 7.21$, $P < 0.01$; Figure 2-4). Inhibition of DNA methylation increased ERα cell number overall, in a pattern that was the mirror image of that seen for effects on calbindin cell number: significant for males and testosterone treated-females ($P < 0.01$ in both cases), with no effect in females. As a result, the magnitude of the sex difference was reduced, although not eliminated, in zebularine-treated animals.

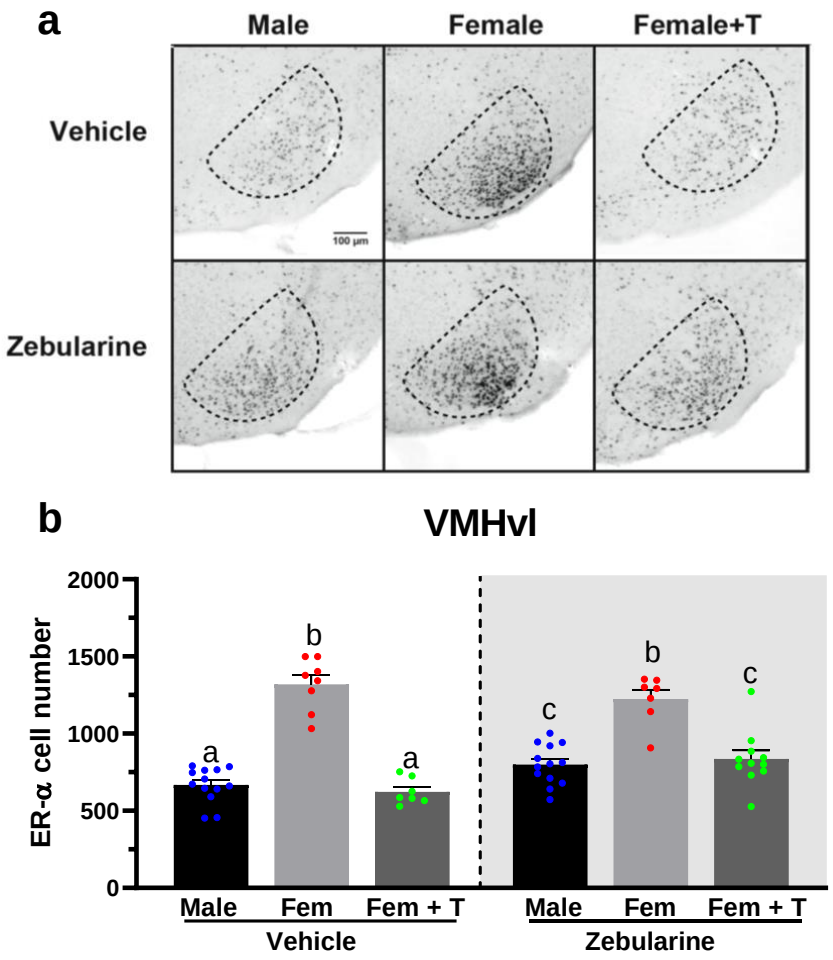

Figure 2-5 Neonatal zebularine increased estrogen receptor α (ERα) cell number in the ventrolateral portion of the ventromedial hypothalamus (VMHvl) of males and testosterone-treated females. *(A) Photomicrographs of ERα cells in the VMHvl at weaning in males, females, and testosterone-treated females that received ICV vehicle or zebularine at birth. (B) Quantification of ERα cell number at weaning demonstrates that vehicle-treated females had more ERα cells than males or testosterone-treated females (Female + T; $P < 0.0001$) There was a significant interaction between group and zebularine treatment, such that neonatal zebularine increased ERα cell number in males and testosterone-treated females, but not in females (a vs c: $P < 0.05$**). *Data are mean ± SEM. N = 5 - 8 per group.*

2.3.4 DNMT inhibition does not alter kisspeptin cell number

As expected, we found a marked sex difference in kisspeptin cell number in the AVPV/PeN of vehicle-treated mice, with many more kisspeptin-positive cells in females (t_{16} = 12.89; $P < 0.0001$). Neonatal testosterone treatment decreased kisspeptin cell number in females (t_{15} = 11.90; $P < 0.0001$) to a level nearly identical to that in males. We did not find evidence of a role for DNA methylation in the development of this sex difference: two-way ANOVA found a significant main effect of group on kisspeptin cell number ($F_{2, 47}$ = 258.6, $p < 0.0001$; Figure 2-6), with no effect of zebularine and no group-by-treatment interaction. There was, however, a trend for increased kisspeptin cell number in zebularine-treated animals ($F_{1, 47}$ = 3.30, $P = 0.076$).

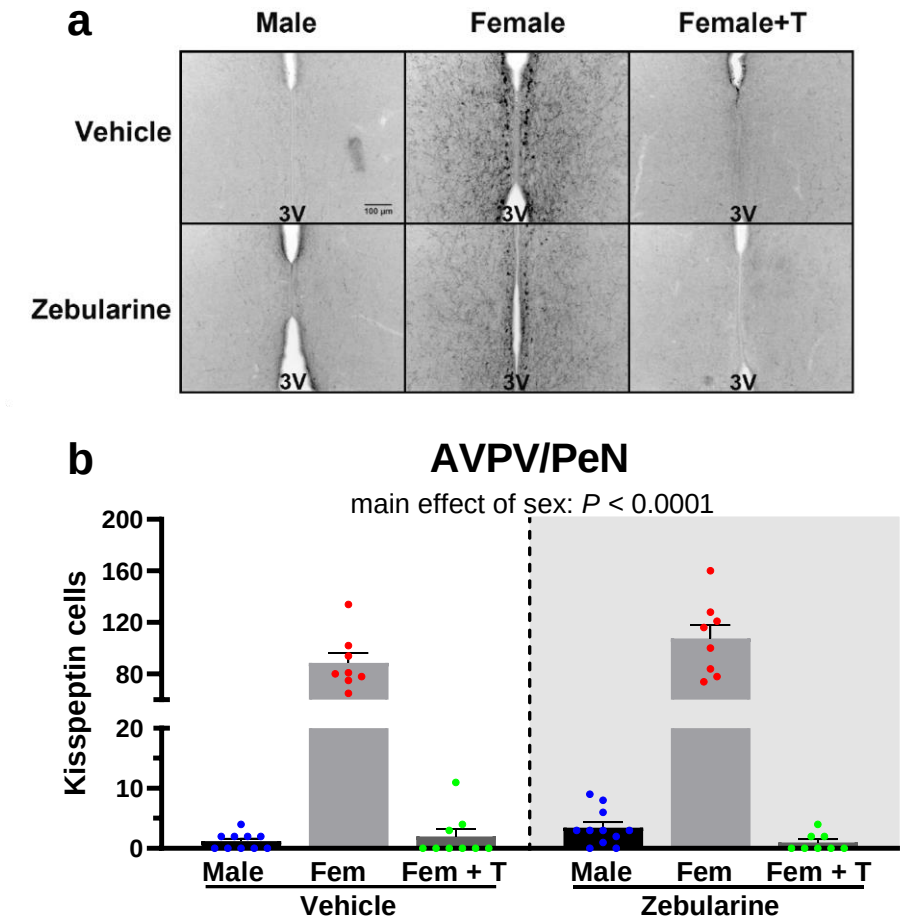

Figure 2-6 Inhibition of DNMT activity at birth did not affect the highly sexually dimorphic group of kisspeptin cells in the anteroventral periventricular nucleus / rostral periventricular region (AVPV/PeN). *(A) Photomicrographs illustrating the sex difference and effects of neonatal testosterone or zebularine on kisspeptin+ cells in the AVPV/PeN. 3V: third ventricle. (B) Quantification reveals that females had 40-fold more kisspeptin-positive cells than did males or testosterone-treated females at weaning (P < 0.0001). Neonatal zebularine treatment did not significantly affect kisspeptin cell number. Data are mean ± SEM. N = 8 – 10 in all groups.*

2.4 Discussion

Neonatal testosterone (or its estrogenic metabolites) can alter DNA methylation patterns in the brain (Schwarz et al., 2010; Ghahramani et al., 2014; Nugent et al., 2015). To test the hypothesis that hormone exposure is "encoded" by changes in DNA methylation, which underlie sex differences in the number of cells expressing phenotypic markers, we inhibited DNMT

activity during the neonatal critical period for sexual differentiation in mice. Our findings support the conclusion that DNA methylation contributes to sex differences in calbindin cell number in the mPOA and BNST, and ERα cell number in the VMHvl, but not to kisspeptin cell number in the AVPV/PeN.

Males have more calbindin-positive neurons than do females in the mPOA and BNST, and treating females with testosterone at birth masculinized both cell groups. Similarly, the neonatal inhibition of DNA methylation increased the number of calbindin cells in both regions only in females, and eliminated the normal sex differences. This suggests that females have neurons in the mPOA and BNST with the potential to express calbindin, but that are prevented from doing so by DNA methylation.

The female-biased sex difference in ERα cell number in the VMHvl at weaning was also completely eliminated by treating newborn females with testosterone and, in this case, neonatal DNMT inhibition increased the number of ERα cells in males and testosterone-treated females, with no effect in control females. Thus, DNA methylation is at least partly responsible for suppressing ERα cell number in males and masculinized females. Because zebularine did not fully increase ERα cell number in males and testosterone-treated females to female-like levels, mechanisms other than DNA methylation may be involved. Alternatively, we achieved only partial inhibition of DNMT activity (a 54% reduction at 6 h), and a more profound inhibition may be required for female-like development of the ERα phenotype. Taken together, our findings demonstrate that DNA methylation underlies both feminization (as shown by calbindin cell number in the present study and Nugent et al., 2015), and masculinization (ERα cell number in the present study and Mosley, Weathington, et al., 2017) of neuronal cell phenotype.

Total DNMT activity was markedly decreased at 6 hours, but not at 24 hours, after neonatal zebularine treatment. Despite the transient suppression, effects on calbindin and ERα cell number were long-lasting (*i.e.*, to at least 3.5 weeks of age). This suggests that early life disruptions in DNA methylation may have programming effects on neuronal phenotype. Patterns of DNA methylation and its counterpart, hydroxymethylation, are dynamic during postnatal development (Schwarz et al., 2010; Szulwach et al., 2011; Lister et al., 2013; Cisternas, Cortes, Bruggeman, et al., 2020). Previous studies have shown that pharmacological perturbations to epigenetic mechanisms do not globally affect the genome, but may particularly target genes undergoing active regulation (Glaser et al., 2003). The present results suggest that this includes genes subject to hormone-dependent sexual differentiation during perinatal life. In the mPOA of rats, sexual differentiation of male copulatory behavior and dendritic spine density remained sensitive to inhibition of DNA methylation as late as postnatal day 10 (Nugent et al., 2015). It will be interesting to determine whether transient epigenomic disruptions later in life would impact neurochemical phenotype or, alternatively, whether there is a perinatal critical window for establishing the number of cells with the potential to express specific markers.

Gonadal steroids may alter DNA methylation by controlling the expression or activity of methylating and demethylating enzymes. For example, females have higher DNMT activity and/or gene expression in the neonatal mPOA (Nugent et al., 2015; Cisternas, Cortes, Bruggeman, et al., 2020), as well as lower expression of the TET enzymes that are responsible for de-methylation (Cisternas, Cortes, Bruggeman, et al., 2020). Thus, the balance is shifted to greater methylation in females. Because calbindin cell number in the mPOA is reduced in females compared to males, the sex differences in enzyme expression/activity are consistent with the canonical effect of DNA methylation to inhibit gene expression.

Other sex differences are not as easy to reconcile with the usual association of DNA methylation with transcription inhibition. For example, females have greater expression than males of some genes in the mPOA (Kühnemann et al., 1994; Yokosuka et al., 1997), and TET enzyme expression is higher in males than in females in the neonatal VMH (Cisternas, Cortes, Bruggeman, et al., 2020), yet males have a reduced number of ERα cells. It is likely that some of the effects of testosterone, or neonatal DNMT inhibition, are due to methylation changes directly on the genes in question, whereas others are indirect. For example, a reduction in DNA methylation may favor the expression of an upstream gene(s) that represses the ERα gene (*Esr1*) in males. Alternatively, a growing number of examples contradict the canonical association of DNA methylation with transcriptional repression, supporting a cell type or genomic context-specific role of DNA methylation (Aran et al., 2011; Bogdanovic et al., 2011; Greenberg & Bourc'his, 2019), and that could be true of the genes encoding the cell-type markers examined here. Methods such as bisulfite sequencing can be used in future studies to determine whether sex differences in cell phenotype correlate with changes in methyl or hydroxymethyl marks in promoter regions of the genes of interest, but it will be much more challenging to demonstrate that any one epigenetic mark (or groups of marks) actually *cause* observed differences in expression or cell phenotype.

We found an enormous, 40-fold sex difference in kisspeptin cell number (female > male) in the AVPV/PeN of weanlings. This is consistent with a previous observation that the sex difference in this region emerges prior to puberty in mice (Clarkson & Herbison, 2006). In rats, the sex difference in kisspeptin cell number in the AVPV/PeN results from early life exposure to testosterone and its estrogenic metabolites (Kauffman et al., 2007; Homma et al., 2009), and our findings confirm a similar mechanism for mice. However, the neonatal inhibition of DNA

methylation had no effect on kisspeptin cell number in males or females, and also did not prevent the masculinizing effect of testosterone in females. Semaan et al. (2012) previously investigated epigenetic mechanisms in the sexual differentiation of kisspeptin cell number in the AVPV/PeN. Although they found a difference in DNA methylation of the *Kiss1* gene promoter between male and female mice, it was in the opposite direction to that expected (lower methylation in males). Moreover, an impairment of CpG-binding protein-2, which binds to methylated DNA to form a repressive complex, did not affect the sex difference in kisspeptin cell number, and an inhibition of histone acetylation in newborn mice also did not reduce the sex difference in kisspeptin in the AVPV/PeN (Semaan et al., 2012). Taken together with the current study, there currently is not compelling evidence linking DNA methylation or histone acetylation to the sex difference in kisspeptin cell number in the AVPV/PeN, although additional studies are clearly needed before either mechanism can be ruled out.

Differences in neurochemical phenotype may be the most common type of sex difference in the nervous system, yet relatively little is known about underlying molecular mechanisms. Our findings suggest that the regulation of neurochemical phenotype by DNA methylation is cell-type specific. The scenario is likely to be even more nuanced than the relatively simple examples examined here. In regions such as the VMHvl, for example, ERα-expressing neurons are not a homogenous cell group, but are comprised of multiple subtypes, with non-overlapping projections and functions (Correa et al., 2015; Chen et al., 2017; Lo et al., 2019; van Veen et al., 2020). Males and females start out with an equally high number of ERα neurons in the VMHvl at birth (Mosley, Weathington, et al., 2017), and the sex difference that emerges by weaning could be the consequence of testosterone-dependent DNA methylation in some, but not all, *Esr1* lineage subtypes in males. Given the crucial role of neurochemistry in neuron function, the

"decision" of a cell to express or not express a given receptor (e.g., ERα), or calcium-binding protein (*e.g.*, calbindin) will have clear functional consequences for the entire neural circuit, as well as the functions and behaviors it controls.

3 DNA METHYLATION AND DEMETHYLATION UNDERLIE THE SEX DIFFERENCE IN ESTROGEN RECEPTOR ALPHA IN THE ARCUATE NUCLEUS

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3.1 Introduction

A transient exposure to testosterone during perinatal life underlies the emergence of many sex differences in the mammalian brain (Lenz et al., 2012; McCarthy et al., 2017). For example, male mice have more neurons expressing calbindin and vasopressin in several forebrain regions (Edelmann et al., 2007; Rood et al., 2013), while females have more neurons expressing estrogen receptor (ER) α (DonCarlos & Handa, 1994; Yokosuka et al., 1997). In some cases, sex differences in cell number are due to differential cell death in males and females (Forger, 2006, 2009a), but other differences persist even when developmental cell death is prevented, e.g., (de Vries et al., 2008; Gilmore et al., 2012). In the latter case, sexual differentiation is often a process of establishing stable, sex-specific patterns of gene expression in neurons, *i.e.*, the differentiation of neurochemical phenotype. Although sex differences in neurochemical phenotype are common, the underlying molecular processes are largely unknown. Several recent findings suggest that epigenetic modifications, which play prominent roles in the differentiation of cell type throughout the body, may underlie sexual differentiation of cell

phenotype in the brain (Nugent et al., 2015; Forger, 2016; Mosley, Weathington, et al., 2017; Manoli & Tollkuhn, 2018; Cisternas, Cortes, Golynker, et al., 2020).

The mediobasal hypothalamus contains prominent populations of ERα expressing neurons in the arcuate nucleus (ARC) and ventrolateral area of the ventromedial nucleus (VMHvl) (Yokosuka et al., 1997; Pérez et al., 2003; Brock, Mees, et al., 2015). ERα neurons in these regions play important, and often sex-specific, roles in the control of feeding behavior, energy homeostasis, sexual behavior, and the regulation of bone density (Y. Xu et al., 2011; Frank et al., 2014; Herber et al., 2019). We recently found that inhibiting DNA methylation in newborn mice reduces or eliminates the usual female bias in the number of neurons expressing ERα in the VMHvl (Mosley, Weathington, et al., 2017; Cisternas, Cortes, Golynker, et al., 2020).

DNA methylation is catalyzed by DNA methyltransferases (DNMTs) and usually occurs at the 5th carbon position of cytosine residues followed by a guanine (CpG sites) (Adams, 1990). DNA methyl marks (5-methylcytosine; 5mC) can be quite long-lasting (M. Kim & Costello, 2017), but can also be removed through a series of oxidative steps catalyzed by ten-eleven translocation enzymes (TETs). In the first step, 5mC is converted to 5-hydroxymethylcytosine (5hmC), which is both an intermediary in demethylation and an independent epigenetic mark in its own right (Bachman et al., 2014). Although a growing number of exceptions are reported (Ball et al., 2009; Rishi et al., 2010; Semaan et al., 2012; Irwin et al., 2014; Yang et al., 2014; Wan et al., 2015), 5mC is typically associated with gene repression, whereas 5hmC is most often associated with gene expression (Bird, 2002; Lister et al., 2009; Irwin et al., 2014; Gross et al., 2015). Thus, the emergence of a particular cell phenotype in part depends on the balance

between methylation (controlled by DNMTs) and demethylation (controlled by TETs) at specific genes.

The methylation landscape of the brain changes dynamically during the first few weeks (rodents) or years (humans) of life (Lister et al., 2013). We recently found peak expression and enzyme activity of DNMTs and TETs in the mouse hypothalamus during the first postnatal week (Cisternas, Cortes, Bruggeman, et al., 2020), which coincides with the critical period for sexual differentiation of the brain and behavior. Furthermore, we found sex differences in *Tet* gene expression in the neonatal hypothalamus, with greater expression of *Tet2* and *Tet3* in males (Cisternas, Cortes, Bruggeman, et al., 2020). Here, we manipulated DNMTs and TETs neonatally to test the hypothesis that DNA methylation and/or demethylation underlie the development of a sex difference in ERα cell number in the ARC. We also examined DNA methylation of *Esr1* promoter regions in the ARC and neighboring VMHvl to determine if sex differences in ERα expression correlate with differences in CpG methylation.

3.2 Materials and Methods

3.2.1 Animals

C57BL6/J mice were bred in our vivarium and checked daily for pups. Mice were housed in cages with corn cob bedding in a room maintained at 22°C with food and water available ad libitum. Animal procedures were performed in accordance with the National Institutes of Health animal welfare guidelines and were approved by the Georgia State University Institutional Animal Care and Use Committee.

3.2.2 Neonatal testosterone treatment and DNA methyltransferase inhibition

The brains used in this portion of the study were the same as those from a prior study reporting on the effects of neonatal inhibition of DNA methylation on ERα in the POA and

VMHvl (Cisternas, Cortes, Golynker, et al., 2020). Male (*n* = 23) and female *(n* = 19) controls received a subcutaneous injection of 25 µL of peanut oil on the day of birth and following day, while androgenized females (*n* = 21) received testosterone propionate (100 µg in 25 µL peanut oil; Millipore Sigma, St Louis, MO). Half of the animals in each of the three hormonal groups also received intracerebroventricular (ICV) injections of either vehicle or a DNA methyltransferase inhibitor, zebularine, on the day of birth (postnatal day (P) 0) and P1. Vehicle (500 nL per hemisphere; 10% dimethyl sulfoxide in 90% saline) and zebularine (300 ng in 500 nL vehicle per hemisphere; Millipore Sigma) were injected into the lateral ventricles of cryoanesthetized pups, as described previously (Cisternas, Cortes, Golynker, et al., 2020). Zebularine is a cytosine analog which incorporates into DNA and traps DNMTs (Zhou et al., 2002). In post-mitotic cells such as neurons, cytosine analogs are incorporated via the base excision repair pathway, a mechanism that replaces entire cytosine bases during active methylation/demethylation cycles (Yamagata et al., 2012; Gavin et al., 2013; Lister & Mukamel, 2015). All animals were tattooed for identification and euthanized prior to puberty at P25. This age was chosen because it allowed us to examine relatively long-lasting effects of the neonatal inhibition of DNA methylation in the absence of confounding effects of post-pubertal gonadal hormones.

3.2.3 Neonatal Tet inhibition

Tet mRNA expression in the hypothalamus is elevated during the first postnatal week, and sex differences in *Tet2* and *Tet3* expression (male > female) are observed during this time (Cisternas, Cortes, Bruggeman, et al., 2020). To test the role of demethylation in the development of the sex difference in ERα expression in the ARC and VMHvl, we used small interfering RNAs (siRNAs) to reduce Tet expression in males to female-like levels. ICV

injections were performed on cryoanesthetized pups as described above. On P5, females (n = 7) received a control injection of non-targeting RNAs in delivery media while males received either the control (n = 15) or a cocktail (n = 14) of SMARTPool siRNAs targeted against *Tet2* and *Tet3* (400-500 pmol; Accell, Horizon Discovery, Cambridge, UK). Each SMARTPool contained a mixture of four siRNAs targeting the same mRNA. Previous studies using Accell siRNAs report a neuron-selective knock-down that is maximal 2-4 days after a single ICV injection (Nakajima et al., 2012; Hazzan et al., 2017). The brains of half of the control- and siRNA-treated males were collected two days after injection (P7), and punches were taken of the anterior and posterior hypothalamus to confirm gene knock-down by RT-qPCR, as described in (Cisternas, Cortes, Bruggeman, et al., 2020). The remaining brains were harvested at P25.

3.2.4 Immunohistochemistry and quantification of labeling

Brains were fixed in 5% acrolein in 0.1 M phosphate buffer for 24 h, then transferred to 30% sucrose for several days. Brains were frozen-sectioned into four series (zebularine-treated animals) or two series (siRNA-treated animals) of 30 μm thickness and stored in cryoprotectant (30% sucrose, 30% ethylene glycol, and 1% polyvinylpyrrolidone in 0.1 M phosphate buffer) until immunohistochemical staining. One series of zebularine-treated tissue and one series from the siRNA experiment were stained for ERα (rabbit anti-ERα, 1:20,000; EMD Millipore, Billerica, MA), as described previously (Cisternas, Cortes, Golynker, et al., 2020). For the zebularine experiment, images through the ARC were captured using Stereo Investigator software (MBF Bioscience, Williston, VT), and ERα labeling was quantified using ImageJ (Version 1.52/1.53; National Institutes of Health, Bethesda, MD). The ARC was identified using well-defined landmarks including the shape of the third ventricle and presence of the median eminence. We drew a contour around the ARC in each section and pixels above background per

μm2 was determined using the Shanbhag algorithm. The four sections through the ARC with the greatest labeling were summed for each animal. To validate this method, we also manually counted ERα cells in a subset of male and female animals using Stereo Investigator and confirmed a very similar pattern of results. For the siRNA experiment, images through the ARC and VMHvl were captured, contours were drawn around each brain region, and pixels above background per μm2 was determined from the seven (ARC) or five (VMHvl) sections with the greatest labeling.

To test whether our treatments had a generalized effect on cell types in the ARC, additional series of sections were labeled for the detection of kisspeptin (rabbit anti-kisspeptin, 1:2,000; EMD Millipore) or calbindin (mouse anti-calbindin-D28k, 1:20,000; Millipore Sigma), as previously (Mosley, Weathington, et al., 2017). The calbindin-labeled tissue included only males and females in the zebularine experiment (*i.e.*, testosterone-treated females were not included) and was counter-stained with thionin to aid in visualization. Brains from a separate cohort of P1 and P25 mice (P1: n = 7 males, 6 females; P25: n = 8 males, 10 females) were also collected and immunostained for ERα to determine if sex differences were present in untreated animals.

3.2.5 Quantification of cell death

To determine whether zebularine treatment altered developmental cell death, brains from a separate cohort of vehicle- and zebularine-treated male (n = 11) and female (n = 9) pups were collected 6 hours after the last injection on P1 and immunolabeled for activated caspase-3 (AC3) as previously described (Ahern et al., 2013). We drew contours around the ARC and counted the number of AC3-positive cells bilaterally. Cell death density was obtained by dividing the number of AC3-positive cells by the area sampled and is expressed as AC3 cells per mm^2.

3.2.6 Stereological cell counts

One series of brain sections from vehicle- and zebularine-treated females was stained with thionin to determine whether zebularine altered the total number of cells in the ARC at weaning. Because zebularine specifically affected ERα in the ARC of females, only females were included in this analysis. Unbiased stereology using the optical fractionator probe was performed using Stereo Investigator software (MBF Biosciences). Contours were drawn around the ARC at low power, and counts of cells exhibiting a neuronal morphology were performed with a 100x oil objective using a 324 μm^2 counting frame and 4,900 μm^2 sampling grid. The Gundersen coefficient of error for counts ranged from 5-7% (Gundersen et al., 1999).

3.2.7 Bisulfite mapping of Esr1 in the ARC and VMHvl

A separate cohort of male (n = 84) and female (n = 84) pups received ICV injections of vehicle or zebularine on P0 and P1. Forty-eight vehicle-treated pups (24 of each sex) were euthanized ~4 h after ICV injection on P1. The 120 remaining animals were collected on P25, and body weight was recorded for about half of these animals (n = 33 males and 35 females, randomly selected). Brains were removed, flash-frozen in 2-methylbutane cooled to -20° C, and cut on a cryostat to the level of the ARC and VMHvl. Punches were taken from the ARC and VMHvl and kept at -80° C until processing. Because a punch of the ARC damages the adjacent VMHvl, and vice versa, separate animals were used for each brain region. Punches from three animals of the same sex were pooled per sample. Bisulfite conversion followed by pyrosequencing was performed by EpigenDx (Hopkinton, MA) to obtain single-nucleotide resolution of modified cytosines in *Esr1*. Specifically, we assessed the modification of cytosines in 16 CpG sites in the untranslated regions of Exons A and C. Because bisulfite sequencing cannot distinguish between 5mC and 5hmC, we refer to the results of this analysis as “total

methylation" (as in (Irwin et al., 2014; He et al., 2020)). Methylation of these regions was previously shown to associate with changes in *Esr1* gene expression in the mouse brain during development or after bisphenol A exposure (Westberry et al., 2010; Kundakovic et al., 2013).

3.2.8 Statistics

Independent, two-tailed t-tests were used to compare ERα labeling in the ARC of untreated males and females on P1. Two-way ANOVAs (group-by-treatment) were used to compare body weight, and ERα, kisspeptin or calbindin labeling at P25 in animals that received either vehicle or zebularine neonatally. Results of pyrosequencing were analyzed using a two-way repeated measures ANOVA for P1 samples, with sex as the between-subjects factor and CpG site as the repeated measure. Pyrosequencing at P25 was analyzed with a three-way repeated measure ANOVA with sex and zebularine treatment as between-subjects factors and CpG site as the repeated measure. A significant main effect of sex was followed by *post hoc* tests of individual CpG sites. Developmental changes in mean methylation across CpG sites were analyzed with two-way ANOVAs (age-by-sex). Effect of *Tet* siRNA treatment on ERα and kisspeptin expression was analyzed by one-way ANOVA. Fisher's LSD or Tukey's *post hoc* test were used where appropriate, and all analyses were performed using Prism Version 9 (GraphPad Software, San Diego, CA).

3.3 Results

3.3.1 A sex difference in ERa cell number in the ARC is programmed by neonatal testosterone and eliminated by an inhibition of DNA methylation

A female bias in estrogen binding, ERα mRNA expression, or ERα immunolabeling in the ARC of rodents has previously been reported, but is not seen in all studies or at all ages (Kühnemann et al., 1994, 1995; Yokosuka et al., 1997; Pérez et al., 2003; Cao & Patisaul, 2011;

Brock, Mees, et al., 2015). We observed prominent ERα labeling in the ARC of untreated animals that appeared confined to cell nuclei (Fig. 3-1). Among untreated mice, we found no sex difference in ERα immunolabeling at P1, but females had more labeling than males at weaning (P25; $t_{16} = 3.64$, $P = 0.002$; Fig. 3-1). In a preliminary study, we also observed greater expression of *Esr1* mRNA in the ARC of control females (n = 4) than of males (n = 4) at P25 using single molecule *in situ* hybridization (Suppl. Fig 3-1).

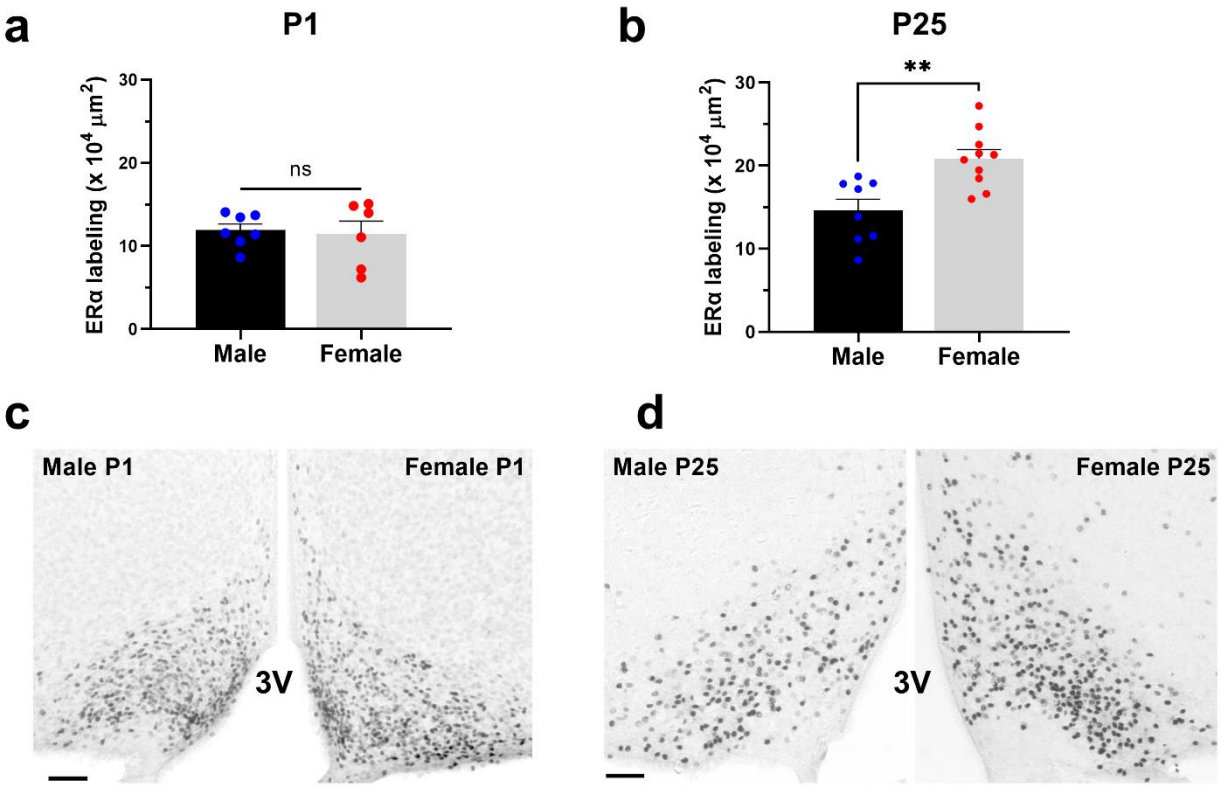


Figure 3-1 A sex difference in ERa labeling in the ARC emerges at weaning. *(A,C) Untreated males and females have similar ERa labeling one day after birth (P1). (B,D) Females have more ERa labeling than males at P25. Mean + standard error of the mean and individual data points are depicted. Scale bar = 50 µm. 3V = third ventricle.*

We next compared ERα in the ARC at P25 in the males, females, and females treated neonatally with testosterone from our previous study to examine the hormonal control of this sex

difference (Cisternas, Cortes, Golynker, et al., 2020). Control females again had greater ERα labeling than did control males ($P < 0.0001$ for Vehicle Female vs Vehicle Male; Fig. 3-2). Neonatal treatment of females with testosterone markedly reduced ERα labeling at P25 ($P < 0.0001$ for Vehicle Female vs Vehicle Female + T), indicating that the sex difference is due to programming effects of testosterone. Our testosterone treatment, in fact, hyper-masculinized females relative to vehicle-treated males ($P < 0.01$).

To test the role of DNMTs in the development of ERα labeling in the ARC, we compared animals that were treated neonatally with zebularine or vehicle. There was no main effect of neonatal zebularine treatment, but a highly significant interaction between group (Male, Female, and Female +T) and treatment (Vehicle vs. Zebularine; $F_{2,54} = 15.12$, $P < 0.0001$; Fig. 3-2). Neonatal zebularine treatment decreased ERα labeling in control females ($P = 0.0005$) to male-like levels, while having no effect on males. Zebularine also slightly increased labeling in testosterone-treated females ($P = 0.035$). As a result, group differences in ERα labeling in the ARC were eliminated among the zebularine-treated animals (Fig. 3-2). The same pattern of results was obtained using direct cell counts of ERα, rather than automated analyses of labeling (not shown).

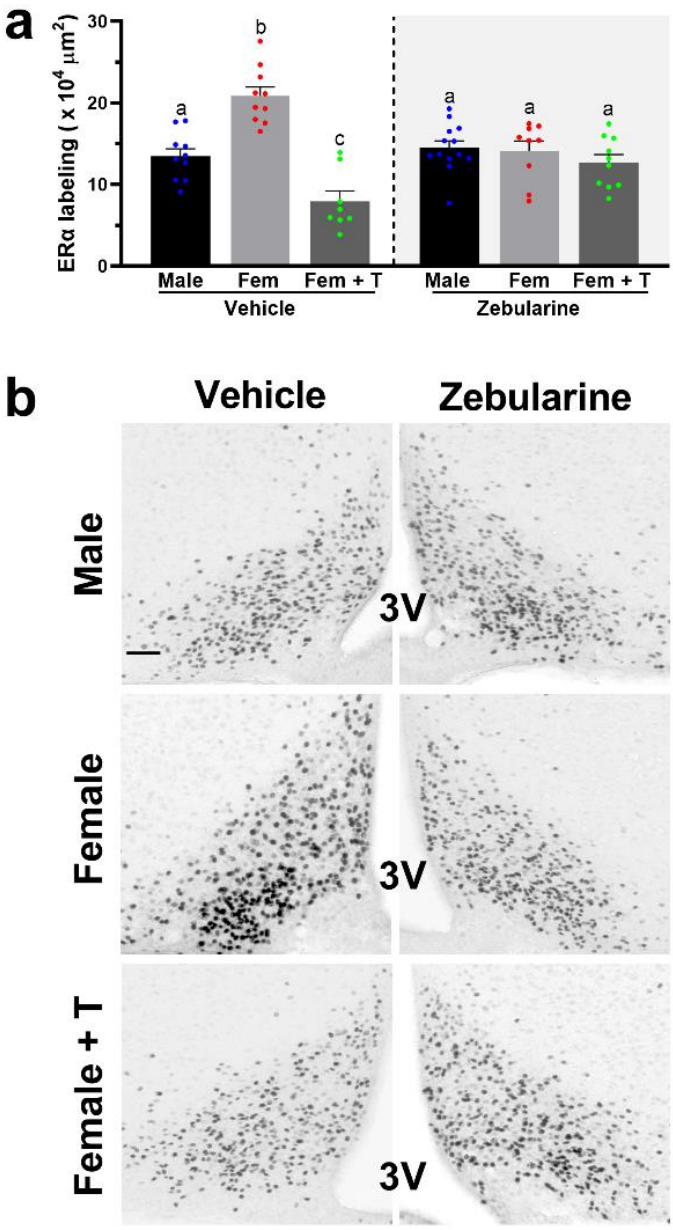


Figure 3-2 The sex difference in ERa labeling in the ARC is programmed by neonatal testosterone and is eliminated by a neonatal inhibition of DNA methyltransferases. *(A) Mean ERa labeling at P25 in control males and females and in females treated with testosterone (T) at birth. Animals also received ICV injections of vehicle (left) or zebularine (right) during the first two days of life. Mean + standard error of the mean and individual data points are depicted. Bars marked by different letters are significantly different (P < 0.01). (B) Photomicrographs showing ERa labeling in the six groups. Scale bar = 50µm.*

Our neonatal treatments overlapped with the peak of developmental cell death in the mouse brain (Mosley, Shah, et al., 2017), but we previously found that effects of zebularine on ERα cell number in the POA are independent of effects on developmental cell death or total cell number at weaning (Mosley, Weathington, et al., 2017). Here, we also found no effect of zebularine on developmental cell death at P1 (Fig. 3-3a), or total cell number at P25 in the ARC (Fig. 3-3b). Thus, neonatal inhibition of DNA methylation reduced the proportion of neurons that express ERα in the ARC of females, rather than the number of surviving neurons.

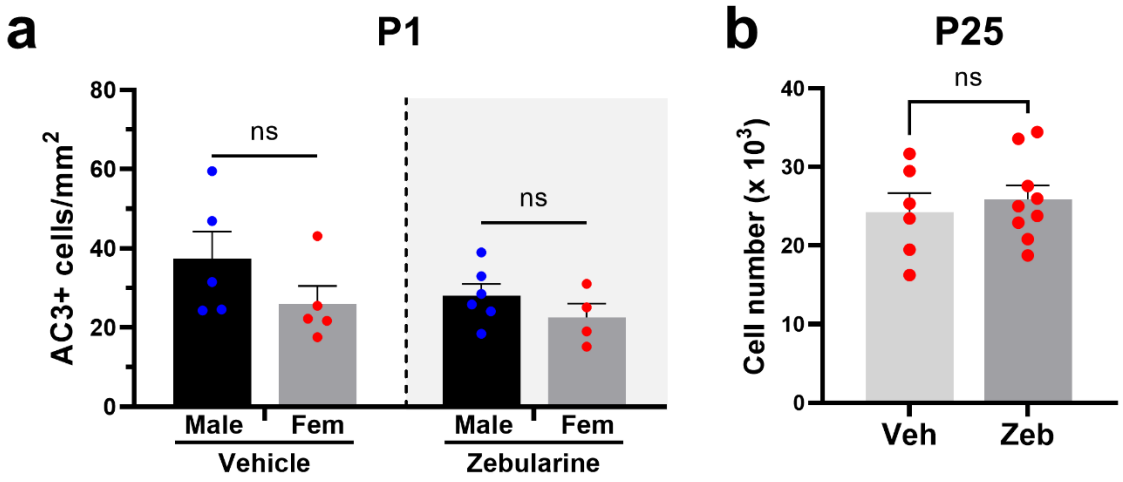


Figure 3-3 Zebularine treatment does not alter cell death on P1 or total cell counts in the ARC at weaning. *(A) Number of cells in the ARC that were positive for activated caspase-3 (AC3) in males and females treated with vehicle or zebularine on P0 and P1. Animals were euthanized 6h after the last injection. (B) Total neuron number in the ARC of P25 females that were treated with vehicle or zebularine neonatally.*

To test whether effects of neonatal zebularine generalized to other cell types in the ARC, we immunohistochemically labeled alternate sections from the animals in this study for kisspeptin and calbindin. We found main effects of sex ($F_{2,49} = 115.2$, $P < 0.0001$) and treatment ($F_{1,49} = 10.88$, $P < 0.0018$), as well as a sex-by-treatment interaction ($F_{2,49} = 4.895$, $P = 0.012$) on kisspeptin labeling in the ARC. Females had more kisspeptin than males ($P < 0.0001$), as

expected (Brock & Bakker, 2013), and labeling was mainly present within fibers (Suppl. Fig 3-2a). Neonatal zebularine treatment reduced kisspeptin labeling of females ($P = 0.002$), but a robust sex difference in kisspeptin remained in zebularine-treated animals ($P < 0.0001$). Females also had more calbindin cells than did males (main effect of sex: $F_{1,26} = 14.31$, $P < 0.001$), but we found no effect of zebularine and no zebularine-by-sex interaction on calbindin cell number in the ARC (Suppl. Fig. 3-2b). Thus, effects of zebularine treatment were cell-type specific, as we, and others, have seen previously (Cheng et al., 2004; Cisternas, Cortes, Golynker, et al., 2020). We also found the expected effect of sex, but no effect of zebularine and no zebularine-by-sex interaction on body weight (Suppl. Fig. 3-2c), suggesting that gross development is normal in zebularine-treated animals, as has also been seen previously by us and others (Mosley, Weathington, et al., 2017; Okhovat et al., 2018).

3.3.2 Down-regulation of Tet expression increases ERa in the ARC of males

DNA methylation is dynamically regulated during brain development (Lister et al., 2013; Schwarz et al., 2010; Szulwach et al., 2011), indicating an active methylation/demethylation cycle. TET enzymes control the turnover of DNA methyl marks, and we previously found higher expression of *Tet2* and *Tet3* mRNA in the POA and mediobasal hypothalamus of neonatal male mice (Cisternas, Cortes, Bruggeman, et al., 2020). To test for a role for DNA demethylation in the development of ERα labeling, we administered siRNAs targeting *Tet2* and *Tet3* to newborn males, while control males and females received injections of non-targeting RNAs. *Tet2*/*Tet3* expression was significantly reduced in punches of the anterior and posterior hypothalamus 48 h after siRNA injection (main effect of siRNA, *Tet2:* $F_{1,10} = 6.72$, $P = 0.027$; *Tet3*: $F_{1,10} = 5.21$, $P < 0.05$; Suppl. Fig. 3-3). The reduction was fairly subtle (~15-30%), but was comparable to the magnitude of sex differences in *Tet* expression seen previously (Cisternas, Cortes, Bruggeman, et

al., 2020). To determine whether this partial knock-down was functionally significant, ERα labeling was examined in the ARC and VMHvl at P25.

We found a significant difference in ERα labeling across the three groups in the ARC ($F_{2,20}$ = 4.66, $P < 0.025$; Fig.3-4). Females again had greater ERα labeling than control males ($P < 0.04$). In males with *Tet2/Tet3* knock-down, ERα labeling was significantly increased relative to control males ($P < 0.015$), to a level very similar to that of females ($P = 0.97$; Fig. 3-4). In contrast, siRNAs against *Tet2 and Tet3* had no effect on ERα labeling in the VMHvl, although we did find the expected sex difference, with greater ERα labeling in females ($F_{2,21} = 14.94$, $P < 0.0001$). To test whether effects of *Tet* downregulation generalized to another cell type in the arcuate, we immunohistochemically labeled the remaining brain sections of these animals for kisspeptin. We found a significant difference in labeling across groups, with females having greater kisspeptin immunoreactivity relative to control males ($P <0.0001$; Suppl. Fig 3-4). In contrast to effects on ERα, however, kisspeptin labeling did not differ between control and siRNA-treated males. Thus, neonatal downregulation of *Tets* appears to disrupt sexual differentiation of specific cell types.

3.3.3 Opposite effects of sex on DNA methylation of Esr1 in the ARC and VMHvl

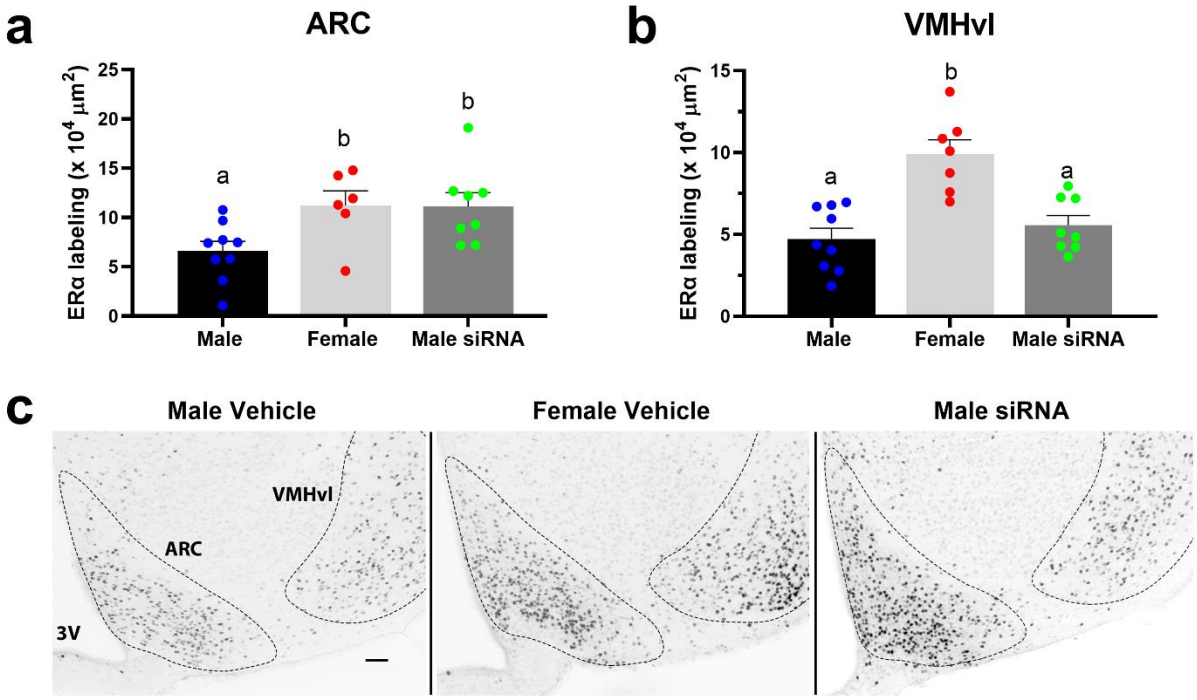


Figure 3-4 Neonatal downregulation of Tets abolishes sex differences in ERα labeling in the ARC, but not VMHvl. *Mean ERα labeling at P25 in control males and females and in males with neonatal Tet2 and Tet3 knock-down in the (A) ARC and (B) VMHvl. Bars marked by different letters are significantly different (P < 0.05). Mean + standard error of the mean and individual data points are depicted. (C) Photomicrographs showing ERα labeling in the ARC and VMHvl in the three groups. Scale bar = 50µm*

A neonatal inhibition of DNA methylation leads to high, female-like ERα expression across groups in the VMHvl (Cisternas, Cortes, Golynker, et al., 2020), but uniformly low, male-like ERα expression across groups in the neighboring ARC of the same animals (current report). Moreover, neonatal inhibition of Tet enzymes, which is expected to increase 5mC, increased ERα in the ARC of males. This suggested that 5mC regulates ER expression differently in the ARC and VMHvl, and may paradoxically increase ERα expression in the ARC. The mouse ERα gene (*Esr1)* can be transcribed from six promoters (untranslated Exons A, B, C, F1, F2, and H)

resulting in five mRNA variants but a single protein product (Kos et al., 2000). Methylation levels at Exons A and C have been associated with changes in gene expression in the brain (Westberry et al., 2010; Westberry & Wilson, 2012). We therefore used bisulfite conversion of DNA followed by pyrosequencing to compare effects of age, sex, and zebularine treatment on total methylation (5mC + 5hmC) of Exons A and C in the ARC and VMHvl, focusing on 16 CpG sites previously shown to regulate expression of *Esr1* in the mouse brain: CpGs 1-11 in Exon A and CpGs 40-44 in Exon C (see Suppl. Table 1) (Westberry et al., 2010; Kundakovic et al., 2013).

The levels of total methylation of *Esr1* in the ARC and VMHvl observed at P1 were similar to those reported by Westberry et al. (Westberry et al., 2010) in the neonatal mouse cortex using the same technique (Fig. 3-5). In Exon A, repeated measures ANOVA indicated no overall sex difference in total methylation across the 11 CpG sites in either the ARC or VMHvl on P1 (Fig. 3-5a). However, small sex differences in both brain regions emerged at P25, in opposite directions: total methylation levels across the 11 CpG sites was significantly higher in females than males in the ARC (main effect of sex, $F_{1, 16} = 8.63$, $P < 0.01$), and higher in males than in females in the VMHvl ($F_{1, 16} = 7.69$, $P < 0.015$; Fig. 3-5a). Analyses of individual CpG sites showed that the pattern (F > M in the ARC, and M > F in the VMHvl) was consistent across most CpG sites (Fig. 3-5b). However, the differences were subtle, and reached significance only for CpG 10 in the ARC, and for CpGs 6, 9, 10, and 11 in the VMHvl (see Suppl. Fig 3-5 for absolute percent methylation values at each CpG site). The effects of sex were also specific to Exon A, as we found no sex differences in Exon C at P1 or at P25 in either the ARC or VMH (all *P*-values > 0.05; Suppl. Fig. 3-6a,b).

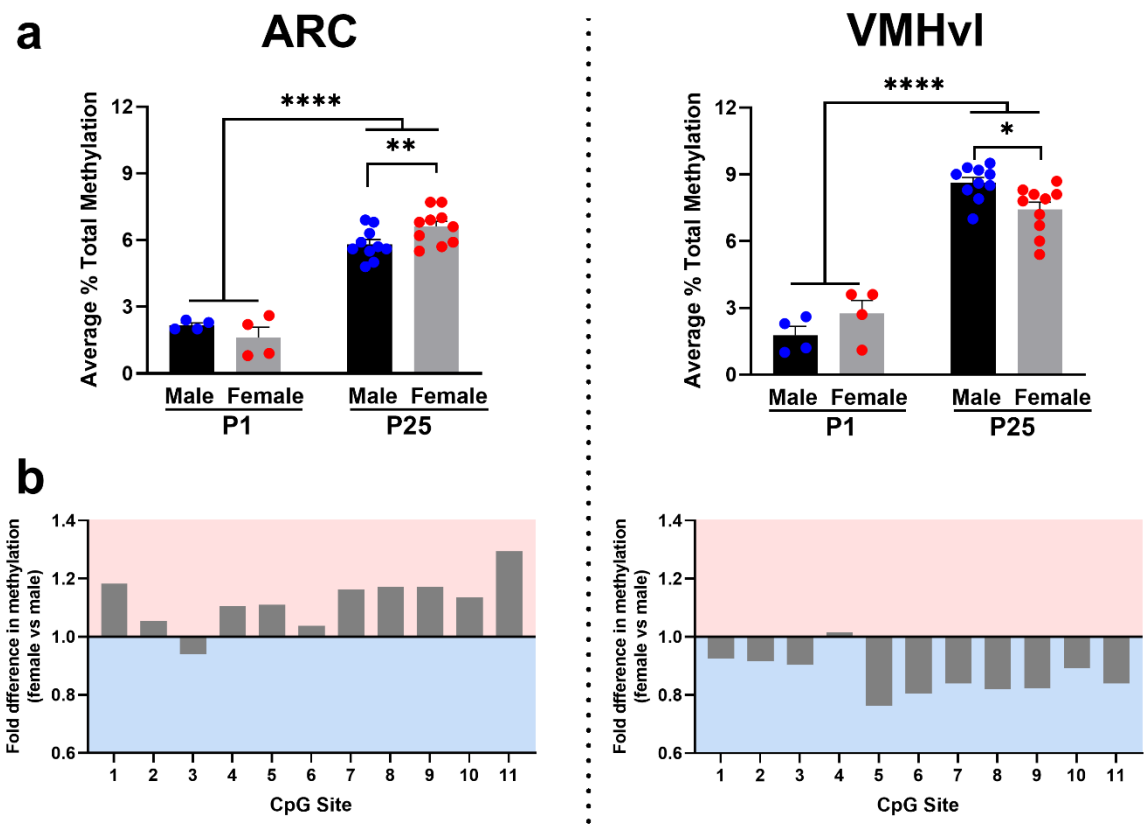


Figure 3-5 Sex differences in total DNA methylation of the Esr1 Exon A promoter emerge at P25, and in opposite directions in the ARC and VMHvl. *(A) Average total percent methylation across all 11 CpG sites in Exon A of the Esr1 promoter in the ARC (left) and VMHvl (right) at P1 and P25 in male and female mice. Overall methylation increased markedly in both brain regions from P1 to P25. There were no significant sex differences at P1. At P25, total methylation was higher in females than in males in the ARC and higher in males than in females in the VMHvl. Mean + standard error of the mean and individual data points are depicted. (B) The sex difference in total methylation for individual CpG sites of Exon A at P25. Fold difference in total methylation is plotted. In the ARC (left), most CpG sites had greater total methylation in females. In the VMHvl, most CpG sites had greater total methylation in males.*

The sex differences that emerged at P25 were superimposed upon large increases in average total methylation of CpG sites of the Exon A promoter region in both sexes and regions between P1 and P25 (main effect of age; $F_{1, 24} > 200$, $P < 0.0001$ in each region; Fig. 3-5), consistent with our previous observation of an overall increase in global 5mC between birth and P25 in the mouse hypothalamus (Cisternas, Cortes, Bruggeman, et al., 2020). In addition, we found an

interaction between age and sex in both regions (ARC: $F_{1, 24} = 5.01$, $P = 0.035$; VMH: $F_{1, 24} = 7.59$, $P = 0.011$): females gained more methylation with age than males in the ARC, and males gained more methylation with age than females in the VMHvl. There was a smaller increase in total methylation with age in Exon C when collapsing across sex (main effect of age; ARC: $F_{1, 24} = 5.41$, $P = 0.029$; VMHvl: $F_{1, 24} = 6.52$, $P = 0.017$; Suppl. Fig. 3-6a,b). Surprisingly, we also found that neonatal zebularine treatment increased total methylation in Exon A at P25 in the ARC ($F_{1,16} = 10.43$, $P < 0.01$; Suppl. Fig. 3-6c), with no sex-by-treatment interaction. *Post hoc* tests indicate that the effect was significant for CpG sites 6 and 9 (not shown). There was no effect of neonatal zebularine treatment on total methylation in the VMHvl.

3.4 Discussion

ERα neurons in the ARC and VMHvl play important roles in estradiol-mediated effects on energy homeostasis, physiology, and behavior. A knockdown of *Esr1* in the ARC increases food intake and bone density in female mice (Y. Xu et al., 2011; Herber et al., 2019), whereas blocking ERα signaling in the VMHvl reduces energy expenditure and thermogenesis (Musatov et al., 2007; Y. Xu et al., 2011; Correa et al., 2015). In both regions, effects are sex-specific, with little or no effect of *Esr1* knockdown in males (Cone et al., 2001; Y. Xu et al., 2011; Frank et al., 2014; Herber et al., 2019). Females have more ERα neurons in the VMHvl (Yokosuka et al., 1997) and some, but not all, previous studies find more ERα protein or mRNA in the ARC of rodents (Yokosuka et al., 1997; Pérez et al., 2003; Cao & Patisaul, 2011). Here, we found greater ERα labeling in weaning age females in three independent cohorts. A similar female bias in ERα cell number has been reported in a variety of brain regions and vertebrate species (Scott et al., 2000; Kruijver et al., 2002; Voigt et al., 2009; Hiraki et al., 2012; Pradhan & Olsson, 2015). In several cases, the sex difference requires differential exposure to testosterone during perinatal

life (Kühnemann et al., 1995; Bakker et al., 1997; Kudwa et al., 2007), but the mechanism(s) by which testosterone programs sex differences in ERα expression, or other sex differences in neurochemistry for that matter, remains largely unknown.

We previously reported that sex differences in ERα in the POA and VMHvl of mice develop postnatally and are epigenetically regulated (Mosley, Weathington, et al., 2017; Cisternas, Cortes, Bruggeman, et al., 2020). Both sexes express high levels of ERα at birth, and ERα cell number decreases in males over the next few weeks. This decrease can be prevented by treating neonatal males with an inhibitor of DNA methyltransferases, with no effect on females and no change in developmental cell death or total cell number (Mosley, Weathington, et al., 2017). We showed here that a sex difference in ERα, favoring females, also develops postnatally in the ARC and is abolished by inhibiting DNMTs during the first two days of life. However, DNMT inhibition eliminated the sex difference in the ARC by decreasing (*i.e.*, masculinizing) ERα expression in females. As previously, zebularine influenced ERα in the ARC without altering neonatal cell death or total cell number, and long-term effects were both sex- and cell-type specific. It is unknown what makes a given gene susceptible to a transient epigenetic disruption. Neonatal zebularine treatment in rats acutely alters the expression of < 2% of all genes in the POA (Nugent et al., 2015) and a similar selectivity has been reported in other studies following treatment with zebularine or other epigenetic inhibitors (Glaser et al., 2003; Cheng et al., 2004; Weaver et al., 2006; Yoo et al., 2008). One hypothesis is that genes actively undergoing regulation at the time of intervention are particularly affected (Glaser et al., 2003; Menegola et al., 2006), and this may include genes undergoing sexual differentiation at birth.

In addition to the sex differences in ERα protein and mRNA at weaning, sex differences in total methylation of a promoter region of *Esr1* emerged at weaning. Across 11 CpG sites of

Exon A, males had a slightly higher percent of total methylation than females in the VMHvl at P25. This is consistent with the hypothesis that 5mC marks may contribute to lower ERα labeling in the VMHvl of males. In the ARC, however, females had a slightly higher percent of total methylation across Exon A, despite their higher levels of ERα protein and mRNA. In addition, neonatal inhibition of DNMTs, which is expected to decrease 5mC, reduced ERα expression in the ARC of females, and neonatal knock-down of *Tet2/Tet3* expression in males, which is expected to increase 5mC, increased ERα cell number at weaning. Taken together, these observations suggest that 5mC is associated with *Esr1* gene activation in the ARC. Although contrary to the canonical association of DNA methylation with gene repression, there are a growing number of cases where DNA methylation of specific gene regions promotes transcription (Ball et al., 2009; Rishi et al., 2010; Semaan et al., 2012; Irwin et al., 2014; Yang et al., 2014; Wan et al., 2015).

One limitation of our findings is that pyrosequencing of sodium bisulfite-treated DNA does not distinguish 5mC marks from 5hmC. The concern in this case is lessened by the fact that 5hmC in neurons primarily accumulates in gene bodies and is depleted in promoter regions (Lister et al., 2013; He et al., 2020). In addition, 5mC is 4-5 times more abundant than 5hmC in CpG dinucleotides (Mellén et al., 2017). Because we examined promoter regions and modifications in the CpG context, most of the total methylation we detected was presumably 5mC. Nonetheless, one would ideally like to sequence using techniques that distinguish 5mC from 5hmC (Yu et al., 2012), although the amount of starting material required is a serious impediment for this type of analysis.

A second limitation is that the sex differences in total methylation we observed were quite small (on the order of 2% in absolute terms and 20% relative differences between sexes

across the CpG sites of Exon A). Although these differences are similar in magnitude to what was previously reported for sex differences in methylation of steroid receptors in the rat POA and mediobasal hypothalamus (Schwarz et al., 2010), it is reasonable to question whether such differences could meaningfully affect gene expression. In our study and related previous studies (Westberry et al., 2010; Kurian et al., 2010; Schwarz et al., 2010), pyrosequencing of *Esr1* was examined from brain punches, which contain many cells types. ERα neurons comprise only a minority of all cells, even in regions such as the VHM and ARC, where they are relatively abundant. Any "signal" (*i.e.*, a sex difference in methylation of *Esr1*) must therefore be detected over quite a bit of noise. Thus, we do not know the methylation status of *Esr1* in neurons specifically expressing ERα or – equally interesting for this study – neurons that expressed high levels of ERα at birth in males but no longer do at P25. Sex differences in those cells could be much larger, or smaller, than sex differences in the aggregate of all cell types that we examined. In clinical studies, very small mean differences in DNA methylation (often, smaller than those reported here) have consistently been associated with disease susceptibility or environmental exposures, *e.g.*, (Heijmans et al., 2008; Y. Liu et al., 2012; Leenen et al., 2016). Subtle differences in methylation have also been associated with shifts in the transcription start sites and resulting changes in mRNA stability and, hence, protein expression of steroid receptors (Leenen et al., 2016). In addition, while small differences in the methylation of a single CpG site may not be meaningful, when hyper- or hypomethylation is spread over several adjacent CpG sites, as was seen here, effects are compounded (Witzmann et al., 2012; Leenen et al., 2016). Ultimately, however, any measure of DNA methylation provides evidence that is correlative in nature. To test whether any given methyl mark *causes* a sex difference in ERα cell number would require specifically manipulating that mark in the cells of interest, and while this is now theoretically

possible (X. S. Liu et al., 2016, 2018), it may be some time before this can be achieved site- and cell-specifically, *in vivo*, in a newborn mouse brain.

In addition to the sex differences in total methylation that emerged at P25, we found that both sexes accumulate modified cytosines in Exon A, and to a lesser degree in Exon C, of *Esr1* from birth to weaning. This is consistent with previous findings of marked increases in 5mC and 5hmC throughout the genome in the mouse hypothalamus and neocortex from birth to adolescence (Cisternas, Cortes, Bruggeman, et al., 2020; Lister et al., 2013). The fact that females have persistently high levels of ERα in both the ARC and VMHvl despite the accumulation of methylation in a brain-relevant *Esr1* promoter, suggests that there are other regulatory mechanisms that allow for gene expression across development, and underscores the difficulty in extrapolating from DNA methylation to mRNA or protein expression.

We expected zebularine, which inhibits DNMTs, to decrease 5mC marks and, therefore, total DNA methylation of *Esr1*, but did not observe that in either the VMHvl or ARC. In the VMHvl, there was no effect of neonatal zebularine treatment on total methylation of Exon A or Exon C, even though this treatment previously led to a lasting increase in ERα in males (Mosley, Weathington, et al., 2017; Cisternas, Cortes, Golynker, et al., 2020). Possible explanations are that zebularine may act indirectly (*e.g*. by inhibiting an inhibitor of *Esr1* in males), or that the *Esr1* promoter regions we examined do not capture the direct effects of zebularine. In addition, recent studies suggest that tissue-specific DNA methylation patterns are often observed outside of CpG islands, for example in CpG shores or "shelves" (Irizarry et al., 2009; Hing et al., 2015). In blood cells of postmenopausal women, decreasing estradiol levels influence CpG *shore* methylation, while sparing promoter regions of *Esr1* (Gardini et al., 2020). Thus, while methylation of promoters A and C have previously been implicated in developmental regulation

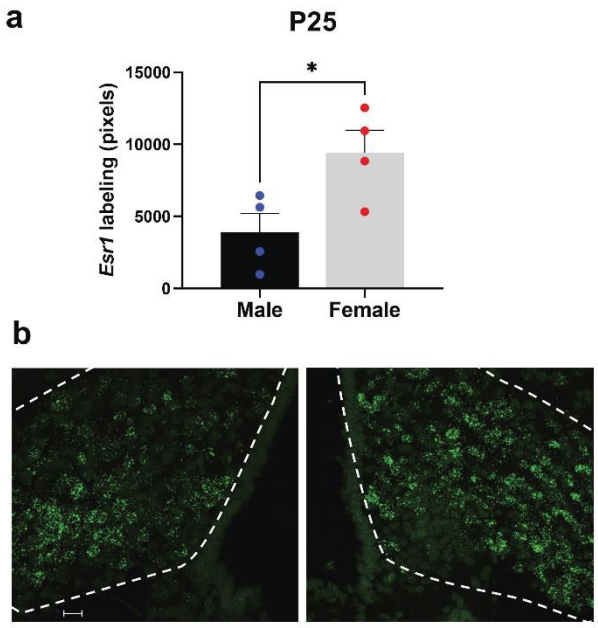
of *Esr1 i*n the brain, methylation in non-promoter regions may also play important roles in regulating gene expression (Kulis et al., 2012; Varley et al., 2013; Yang et al., 2014).

Even more surprising, neonatal zebularine treatment slightly increased the percent total methylation in Exon A of *Esr1* in the ARC at P25. If 5mC indeed activates *Esr1* in the ARC, the increased total methylation after zebularine would be consistent with the increased ERα seen in testosterone-treated females that received zebularine. However, the effect of zebularine was seen across all groups, which is harder to explain. We note that in *post hoc* analyses, the individual CpG sites affected by zebularine did not overlap with those that showed a significant sex difference in the ARC. In the current study, for example, CpG-10 was significantly different by sex, but not by zebularine treatment, in both the ARC and VMHvl. Differences in *Esr1* expression between peripheral tissues have been attributed to the methylation status of a single CpG site (Fürst et al., 2012), although this is uncommon.

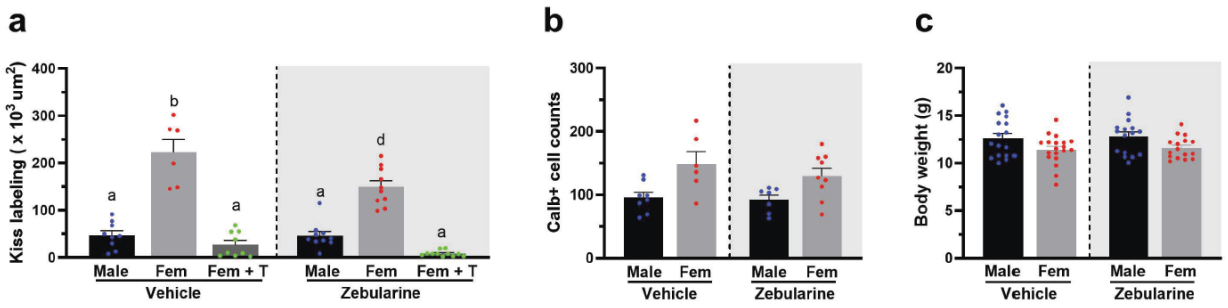
DNA demethylation and 5hmC marks are just beginning to be explored in the context of sexual differentiation. Given its unusually high abundance in gene bodies of neurons (Kriaucionis & Heintz, 2009), 5hmC is likely to have an important role in shaping neural gene expression. We recently found more consistent sex differences in the expression of *Tets* than of *Dnmts* in the neonatal mouse brain (Cisternas, Cortes, Bruggeman, et al., 2020), with higher *Tet2* and *Tet3* expression in males. The fact that suppressing *Tet2* and *Tet3* expression in males increased ERα in the ARC to female-like levels indicates that this sex difference is functionally meaningful, and gives further credence to the idea that the sex difference in ERα expression in the ARC is related to levels of DNA methylation early in development. More generally, this finding suggests that demethylation, or possibly stable 5hmC marks, contribute to sexual differentiation of the brain.

Overall, our data confirm a sex difference in ERα cell number in the ARC at weaning (female > male) that depends on neonatal testosterone. We further show that neonatal inhibition of DNA methylation decreases ERα labeling in females and neonatal inhibition of demethylation increases ERα labeling in males. Females also had slightly higher methylation in a promoter region of *Esr1* at weaning than did males, which was opposite to the pattern seen in the VMHvl. It is possible that neurons with the potential to express ERα are normally inhibited from doing so by the presence of DNA methyl marks in the VMH and the absence of such marks in the ARC, although additional studies would be required to demonstrate this. Since sex differences in both regions are due to perinatal gonadal steroids, testosterone may program ERα cell number through epigenetic mechanisms. In cancer cells, total methylation levels are reduced in response to estradiol (El-Maarri et al., 2011), and one mechanism for this hormone-dependent hypomethylation is the upregulation of *Tet2* expression and co-binding at ER-target genes (Wang et al., 2018; Broome et al., 2021). Neonatal estradiol also dampens the catalytic efficiency of DNMTs in the POA of the rat brain (Nugent et al., 2015). Finally, gonadal steroids alter histone modifications (H.-W. Tsai et al., 2009; Fortress & Frick, 2014) which may, reciprocally, affect DNA methylation levels (Vaissière et al., 2008; Cedar & Bergman, 2009). Thus, there are likely to be multiple parallel and interacting epigenetic mechanisms by which sex steroid hormones orchestrate stable changes in neurochemistry in the developing brain.

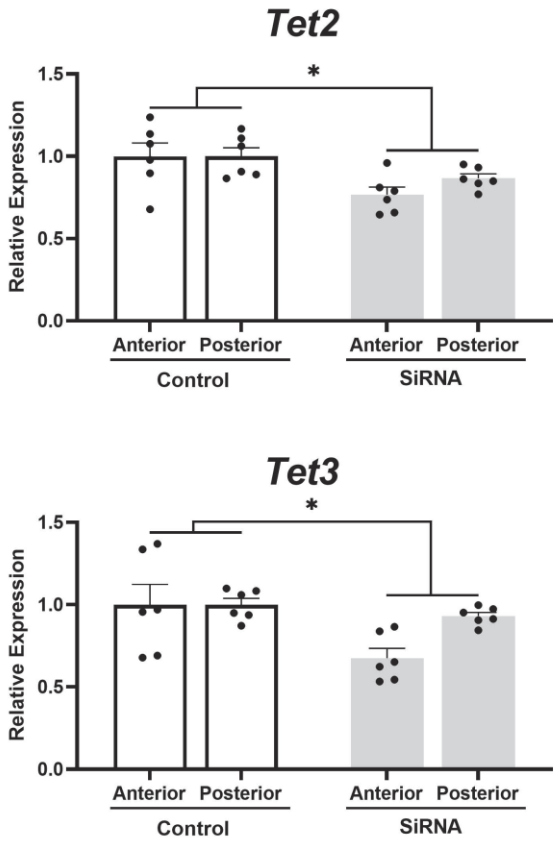
3.5 Supplementary Figures



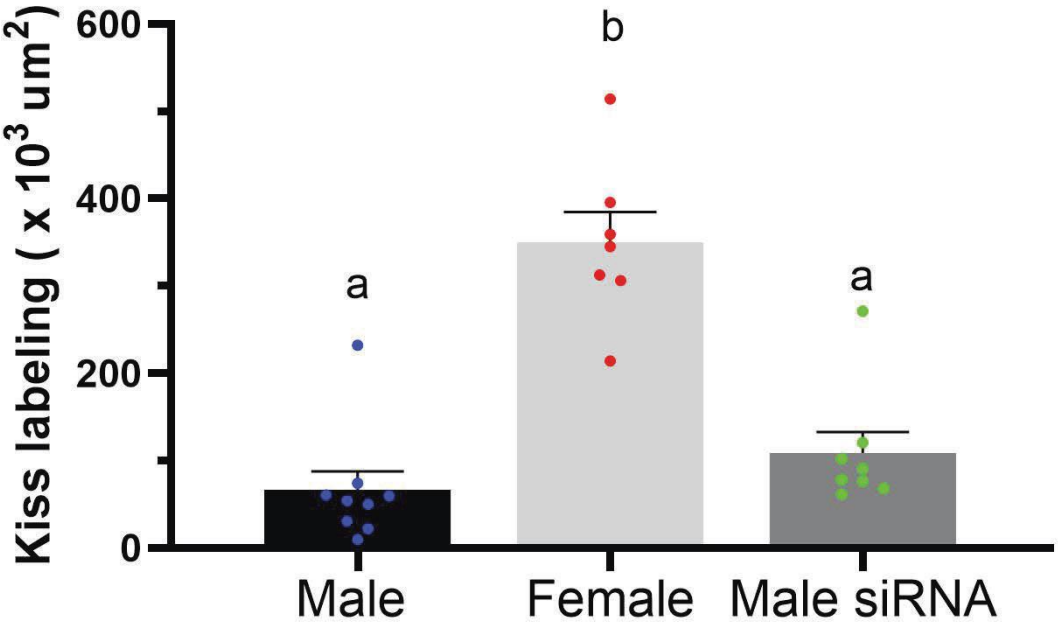
Supplementary Figure 3-1 Females have greater Esr1 mRNA expression in the ARC than males at P25. *The RNAscope Fluorescence Multiplex V2 assay (Advanced Cell Diagnostics, Newark, CA) was used to detect Esr1 in two sections through the mid-arcuate nucleus in vehicle control male and female mice (n = 4 per sex), following the protocol provided by the manufacturer. All animals were from the control group of a separate, ongoing study and had received ICV injections of vehicle (90% saline, 10% dimethylsulfoxide) at birth. (A) The total number of pixels above background in each section was recorded and summed. Mean + standard error of the mean of total pixels and individual data points are depicted. (B) Photomicrographs showing Esr1 labeling in the ARC of a vehicle-treated male (left) and female (right). Scale bar = 20µm.*



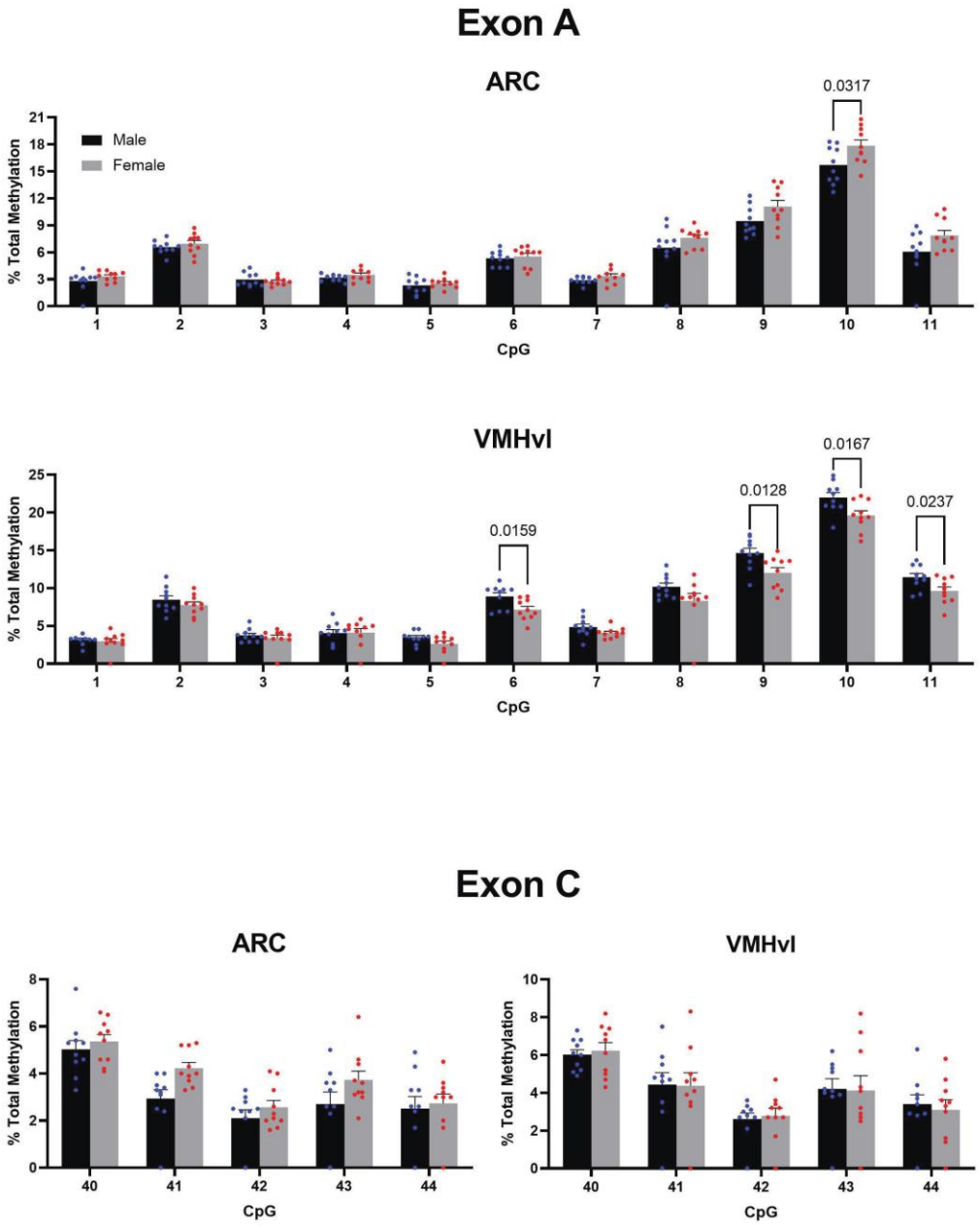
Supplementary Figure 3-2 ***Neonatal zebularine did not eliminate sex differences in kisspeptin or calbindin immunohistochemistry in the ARC or of body weight at weaning.*** *(A) Mean kisspeptin labeling at P25 in control males and females and in females treated with testosterone (T) at birth. Animals also received ICV injections of vehicle (left) or zebularine (right) during the first two days of life. There was a main effect of treatment ($F_{1,49}$ = 10.88, P = 0.002), group ($F_{2,49}$ = 115.2, P < 0.0001), and an interaction ($F_{2,49}$ = 4.9, P = 0.012). Zebularine treatment reduced kisspeptin labelling in females, but did not eliminate the sex difference. N per group: male vehicle (9), female vehicle (6), female + T (9), male zebularine (10), female zebularine (10), female + T zebularine (11). (B) Number of cells in the ARC that were positive for calbindin in males and females treated with vehicle or zebularine on P0 and P1. There was a main effect of sex ($F_{1,26}$ = 14.31, P < 0.01), but no effect of treatment and no sex-by-treatment interaction. N per group: male vehicle (8), female vehicle (6), male zebularine (7), female zebularine (9). (C) There was a main effect of sex, but no effect of zebularine treatment on body weight at P25. N per group: male vehicle (17), female vehicle (19), male zebularine (16), female zebularine (16). Mean + standard error of the mean and individual data points are depicted.*



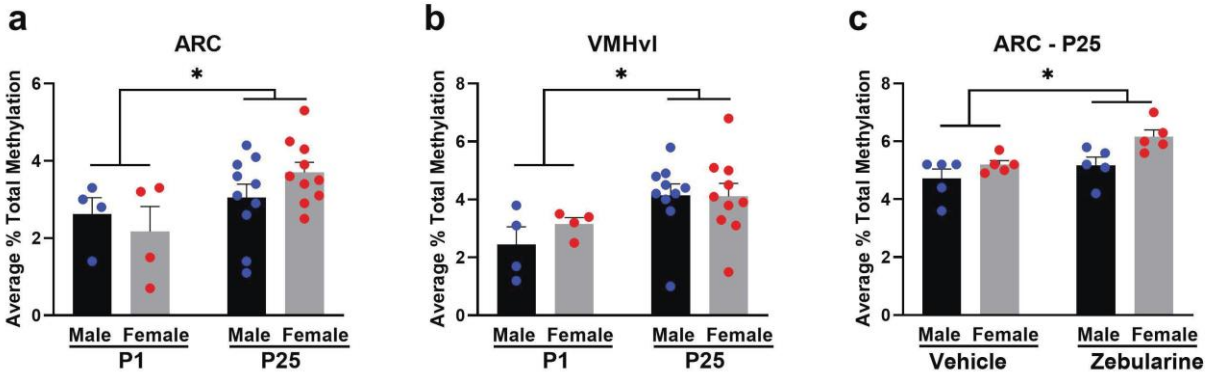
Supplementary Figure 3-3 Confirmation of Tet2 and Tet2 knock-down 48 hours after siRNA intracerebroventricular injection. *There was a modest, but significant reduction in Tet2 (top) and Tet3 (bottom) expression in tissue punches from the anterior and posterior hypothalamus (main effect of siRNA treatment, with no interaction). Mean + standard error of the mean and individual data points are depicted. N per group: control (6), siRNA (6) per region.*



Supplementary Figure 3-4 Neonatal downregulation of Tets does not alter kisspeptin labeling in the ARC at weaning. *Mean kisspeptin labeling at P25 was higher in control females than in males ($F_{2, 21} = 31.30$, $P < 0.0001$ for main effect of group), and neonatal Tet2 and Tet3 knock-down did not alter kisspeptin labeling in males. Mean + standard error of the mean and individual data points are depicted. Bars marked by different letters are significantly different in post hoc tests. N per group: male control (9), female control (7), male siRNA (8).*



Supplementary Figure 3-5 Total methylation of the Esr1 promoter in male and female mice at weaning. *Absolute percent total methylation at each CpG site in exon A and exon C in the* ARC and VMH of males (N = 10) and females (N = 10) at P25. Mean + standard error of the mean and individual data points are depicted. CpG sites with significant sex differences are shown.



Supplementary Figure 3-6 (A,B) There were no sex differences in average total methylation in Exon C of Esr1, but there was an effect of age. *Average total methylation across 5 CpG sites in Exon C of the Esr1 promoter in the ARC (A) and VMHvl (B) at P1 and P25 in male and female mice. Mean + standard error of the mean and individual data points are depicted. There were no significant sex differences at P1 or P25 in the ARC (left) or VMHvl (right).* ***(C) Zebularine increases total methylation in Exon A of Esr1 in the ARC.*** *Average total methylation across 11 CpG sites in Exon A of the Esr1 promoter in the ARC was calculated for each animal. Zebularine-treated males and females had greater total methylation than vehicle-treated animals.*

Table 1

Promoter	From ATG	From TSS	# of CpGs
Exon C	-2163 to -2220	+98233 to +98176	6
Exon A	-131 to -15	+100265 to +100381	11

Promoter Region Exon C

chromosome:GRCm38:10:4709800:4710801:1

AGGAAGAAGATATCCTGGCATAGTTTATAAACTTGATCTCTGCACACTTTGACTGGCATTCTAGTTTATCTGTGAGC
TGTGGTTCACAGCCATCTCAGTTTCCCTCTCCATAGAAAGCACCGGGACATTTCTGGCTCTACAGGTAGGGAGCAAA
GGGGGCTGGAGTTTCTTCTAGGAATGCTGATTCTAGCGGGGCTACTGCTGTCCCTCAGCAGACAGCAAGGCTCCCGG
ACTCCCGCTGCCATTCATCCAGCTCGTGCAGGAGCCCAGCTGCCTTGTCTGCCGGGAGGGGCTGCCAAGTGCCCTGC
CTACTGGCTGCTTCCCGAGAGTCCCTGCCACTCCACATACAAACACATCCACACA**CG**[40]CTCTGCCTTGATCACACAC
CG[41]**CG**[42]CCACT**CG**[43]ATCATT**CG**[44]AGCACATTCCTTCCTTC**CG**[45]TCTTACTGTCTCAGCCCTTGACTTCTACAAACC
CATGGAACATTTCTGGAAAGACGCTCTTGAACCAGCAGGGTAGGATCGGCTTTTGATTTCTCTCTCTTTCTCTCC
TGTAGCTTGAGCGATTTGAGAAAGCAACTTACCTTTCTGCTTAGTGTCTGTCTCCTAGCTGGAGAAGGAAGCTGTGC
TGTTTTTCTATGGGGGGAAATCTGTGCGGCTTTTTTTTTTTTTTTCCCGCTTTCCGGACTTGTAGGATTCTCTGTG
CCATTTATATATAATTTGGCAGGCTCAGATTTTTAAAGAGTCTCATCTGAAGTGCTTGCTTTTGCATGTGTTTTTTA
AAAGGCATTTGAAAATTGAAAGTGTGATTTATGGGAACTAAATCATCTACGAAAAAATTGCTCTAGAAAGTCATGGT
GCTGGCCATAAAGAGAAATATCTGCGATTCACCTAATGTATTTTTAACCCTTCCTTTGCTGTCAATGATACTTATGG
GTGCTAAAACTCATCTTTGTCTTTAGATTTAAGCCAGCTTAGCTGTGGTTTATCCAAAATTATCTGTTTTTCTAAGA
ATTAG

Promoter Region Exon A

chromosome:GRCm38:10:4712200:4713001:1

TCTGCAGGATAGCTCTGCCCCGCAGGGGCAGAGGCAGGGCCAGGGCCAGTACT**CG**[11]TGCCAAGGGGGACTTG**CG**[10]CT
G**CG**[9]CCTTCTCTAAT**CG**[8]CAGGCTCTACTCTTTTTTCCAGGTGGCCCA**CG**[7]**CG**[6]CTGCTGAGCCCTCTG**CG**[5]TG**CG**[4]**CG**[3]
GGGAGCCAGTCTGTAACT**CG**[2]C**CG**[1]GCTGCCACTTACCATGACCATGACCCTTCACACCAAAGCCTCGGGAATGGCCT
TGCTGCACCAGATCCAAGGGAACGAGCTGGAGCCCCTCAACCGCCCGCAGCTCAAGATGCCCATGGAGAGGGCCCTG
GGCGAGGTATACGTGGACAACAGCAAGCCCACTGTGTTCAACTACCCCGAGGGCGCCGCCTACGAGTTCAACGCCGC
CGCCGCCGCCGCCGCCGCCGCCTCGGCGCCGGTCTACGGCCAGTCGGGCATCGCCTACGGCCCCGGGTCGGAGGCGG
CCGCCTTCAGTGCCAACAGCCTGGGGGCTTTCCCCCAGCTCAACAGCGTGTCGCCTAGCCCGCTGATGCTGCTGCAC
CCGCCGCCGCAGCTGTCTCCTTTCCTGCACCCGCACGGCCAGCAGGTGCCCTACTACCTGGAGAACGAGCCCAGCGC
CTACGCCGTGCGCGACACCGGCCCTCCCGCCTTCTACAGGTACCCGCGCCACATCGGGGTGGCCGCTGTGTAAGGCC
TGGCCGGGGGAGGGAGCGCACGAGGGGGGTCACCGCGGGCTGCAGGTCTCCCCATCCCAGCCCTCGGGGAAGTCAGA
AAGGATTCTTTGGCAGCTTTAAAACAACAGTAACAACAC

4 SEXUAL DIFFERENTIATION OF ESTROGEN RECEPTOR ALPHA SUBPOPULATIONS IN THE VENTROMEDIAL NUCLEUS OF THE HYPOTHALAMUS

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4.1 Introduction

Structural sex differences in the brain (*e.g.*, differences in volume, overall neuron number or synapse number in a given region) have garnered a great deal of attention in the sexual differentiation field since their discovery in the 1970s (Gorski et al., 1978, 1980; Nottebohm & Arnold, 1976; Breedlove & Arnold, 1980; Hines et al., 1985, 1992). However, this type of sex difference may be relatively rare, whereas differences in gene expression are widespread. A recent survey of gene expression in 44 tissues throughout the body and brain in humans finds that sex differences are "ubiquitous, but small" (Oliva et al., 2020). Sex differences in neural gene expression are typically restricted to sub-populations of cells within bulk tissue and therefore may be greater in magnitude among specific cell types when examined histologically or at the single-cell transcriptome level.

For example, male mice have many more calbindin immunoreactive cells in the preoptic area (Edelmann et al., 2007) and vasopressin cells in the bed nucleus of the stria terminalis (de Vries & Panzica, 2006; Rood et al., 2013), whereas females have more kisspeptin cells in the anteroventral periventricular nucleus (Semaan et al., 2010). The mechanisms driving these sex differences remain unclear but appear to be independent of cell death. Mice with a deletion of the *Bax* gene do not undergo developmental neuronal cell death and structural sex differences (*i.e.* in volume or overall neuronal number) in several neural regions are eliminated. Nonetheless, *Bax* knockout mice retain sex differences in the number of kisspeptin-, vasopressin-, and calbindin-

expressing cells (de Vries et al., 2008; Semaan et al., 2010; Gilmore et al., 2012). All three sex differences also develop before puberty and therefore do not depend on circulating levels of gonadal hormones; we refer to these as sex differences in "neurochemical phenotypes" – or stable differences in patterns of gene expression.

One well-studied brain region that houses many sex differences in gene expression is the ventrolateral subdivision of the ventromedial hypothalamus (VMHvl). Here, females across many species, including humans, have more neurons expressing the estrogen receptor α (ERα) and/or its gene transcript (*Esr1*) compared to males (Yokosuka et al., 1997; Scott et al., 2000; Kruijver et al., 2002; Beck & Wade, 2009; Cao & Patisaul, 2011). ERα neurons in the VMHvl regulate diverse behavioral and physiological processes, some of which are sex specific. For example, optogenetic stimulation of cells in the VMHvl that express ERα induces defensive behaviors and aggression in both sexes (H. Lee et al., 2014; Hashikawa et al., 2017; Wang et al., 2019), whereas *Esr1* downregulation or developmental ablation of ERα neurons in the VMHvl disrupts physical activity and energy expenditure only in females (Musatov et al., 2007; Y. Xu et al., 2011; Correa et al., 2015).

The heterogeneity of *Esr1* neurons in the VMHvl likely contributes to their diverse functionality. Analyses of immediate early gene expression and electrophysiological recordings suggest that separate groups of VMHvl ERα cells are activated in aggression versus in mating behavior in mice (D. Lin et al., 2011; Hashikawa et al., 2017). Using a single cell sequencing approach, Correa and colleagues recently identified six major subsets of *Esr1* neurons in the VMHvl, each of which may subserve different functions (van Veen et al., 2020). These distinct, but inter-mingled, clusters of *Esr1*-expressing cells are defined by their co-expression of tachikynin1 (*Tac1*, precursor for the secreted molecules, substance P and neurokinins), reprimo

(*Rprm*, first identified as a tumor-suppressor gene involved in p53-dependent arrest of the cell cycle; Ohki et al., 2000), prodynorphin (*Pdyn*, proteolytically cleaved to produce secreted opioid peptides), hippocalcin-like protein 1 (*Hpcal1*), galanin (*Gal*), or somatostatin (*Sst*). Adult female mice have greater *Tac1* and *Rprm* expression in the VMHvl, as well as more ERα cells co-expressing *Tac1* or *Rprm* than do males (van Veen et al., 2020). Ablating these cells by genetic engineering or reducing expression with short-interfering RNAs alters body weight and physical activity (*Tac1/Esr1* cells; Correa et al., 2015) or thermogenesis (*Rprm* cells; van Veen et al., 2020) in females, but not in males. In contrast, adult male mice have greater *Pdyn* expression and more ERα cells in the VMHvl that co-express *Pdyn* (van Veen et al., 2020), although the function of this population of cells is not yet known.

We previously observed high expression of ERα in the VMHvl on the day after birth in both male and female mice (Mosley, Weathington et al., 2017). Females maintain these high levels, but males have many fewer ERα immunoreactive neurons at weaning (Mosley, Weathington, et al., 2017; Cisternas, Cortes, Golynker, et al., 2020), suggesting that about half of the ERα neurons in the VMHvl of neonatal male mice "turn off" ERα expression over the next few weeks. Because this sex difference develops prior to puberty, it is likely not due to activational (*e.g.*, pubertal/adult) hormones and instead suggests developmental programming of neurochemical phenotype. *Tac1* and *Pdyn* cells in the VMHvl (independent of *Esr1* expression) have been implicated in puberty onset, lordosis, and perinatal neuroendocrine regulation (Dornan et al., 1987; Szeto, 2003; Maguire et al., 2017). Not much is known about the function of *Rprm* in the brain outside of its recently discovered role in thermogenesis (van Veen et al., 2020). *Tac1* and *Rprm* expression have been examined in the "four-core genotype" mouse model, in which the effects of the sex chromosomes and the gonads can be studied independently. This work

suggests that the sex differences in *Tac1* and *Rprm* are not due to sex chromosome complement or to gonadal hormones circulating in adulthood (van Veen et al., 2020). Instead, these negative findings strongly suggest that the sex differences in *Tac1* and *Rprm* are due to the programming effects of gonadal steroids early in life, although this has not been directly tested, nor is it known at what point in development the sex differences emerge.

The goals of this study were therefore three-fold. First, to test whether the pattern of *Esr1* gene expression in the VMHvl mirrors the developmental pattern seen previously for ERα protein (*i.e.*, no sex difference at birth, followed by a significant decline in males prior to puberty). Secondly, to test whether sex differences in *Tac1*, *Rprm*, and *Pdyn* are present at birth and/or at weaning. Finally, given the greater expression of *Tac1* and *Rprm* in adult female mice and the female-specific roles of *Tac1*/*Esr1* and *Rprm*/*Esr1* co-expressing cells, we hypothesized that these sub-populations of ERα cells may be preferentially reduced in males between birth and weaning. To address these questions, we used single-molecule fluorescent *in situ* hybridization and analyzed the number of cells expressing *Esr1, Tac1, Rprm, and Pdyn*, as well as the number of *Esr1* cells co-expressing each of the other markers in the VMHvl of newborn and weanling mice.

4.2 Materials and Methods

4.2.1 Animals

C57BL/6J mice were housed in cages with corn cob bedding in a room maintained at 22°C with food and water available ad libitum. Mice were bred in our vivarium and checked daily for pups. Animal procedures were performed in accordance with the National Institutes of Health animal welfare guidelines and were approved by the Georgia State University

Institutional Animal Care and Use Committee. All animals had received intracerebroventricular (ICV) injections of 500 nL of vehicle (10% dimethyl sulfoxide in 90% saline) per hemisphere on postnatal day (P) 1 as part of another study and were tattooed for identification. Mice were euthanized four to six hours after the vehicle administration on P1 or prior to puberty at P25 (*n* = 9-13 per group). Brains were flash-frozen in isopentane and stored at -80 °C until cryo-sectioning.

4.2.2 Single-molecule in situ hybridization

Brains were sectioned coronally on a cryostat into four (P1), or six (P25), 16µm series. The first series was fixed in 4% PFA for 15 minutes, washed in 0.01M phosphate buffered saline, and stained with Hoechst 33342 solution (ThermoFisher Scientific, MA, USA; cat #: 62249) to label cell nuclei. Nuclear staining was used to anatomically identify and match sections through the caudo-lateral VMHvl (Figs. 43–47 in Paxinos & Franklin, 2001), where the largest population of *Esr1* cells is located, for single molecule in situ hybridization. Tissue underwent processing according to the manufacturer's instructions for the RNAscope Multiplex Fluorescent Detection Kit V2 (Advanced Cell Diagnostics, CA, USA). Briefly, tissue was fixed in 4% paraformaldehyde, dehydrated using several dilutions of ethanol, and treated with hydrogen peroxide and RNAscope protease IV solution. RNA target probes against ERα transcript variant 1 (*Esr1*, Cat # 432861), tachykinin 1 (*Tac1*, Cat # 410351) and prodynorphin (*Pdyn*, Cat # 318771) were applied to one series, while probes against *Esr1* and reprimo TP53-dependent G2 arrest mediator candidate (*Rprm*, Cat # 466071) were applied to a second series. Positive and negative 3-plex control probes provided in the kit were also used per manufacturer recommendations. After probe hybridization, amplification solutions (AMP1, AMP2, AMP3) and fluorophores, including Opal 520 (494/525 nm; Cat # FP1487001KT), 570 (550/570 nm; Cat

FP1488001KT), and 690 (676/694 nm; Cat # FP1497001KT; Akoya Biosciences, MA, USA) were applied to the first series, and Opal 520 and 690 were applied to the second series, all at 1:1000-1:1500 dilution. Signal was developed using RNAscope HRP C1, C2, C3 solutions. Nuclei were stained with DAPI and slides were coverslipped using ProLong Gold Antifade (ThermoFisher Scientific; Cat #: P36930). We processed 4-10 animals per sex and age as described above. However, tissue with poor morphology was omitted from further analysis, and this particularly affected neonatal brains, resulting in final group sizes of n = 2-7 for neonates and n = 5-9 for juveniles.

4.2.3. Imaging and quantification

Multiple images of each section were captured using a Zeiss LSM700 laser scanning confocal microscope at 20x (excitation/emissions: 510/510 nm (blue), 639/660 (red), 594/594 nm (green), 647/647 nm (magenta) and stitched together using the Imaris Stitcher (Oxford Instruments, Abingdon, Oxfordshire, UK). The VMHvl was delineated by a contour, and cells outside of the contour were masked using Imaris (version 9.5, Oxford Instruments). Nuclei were labeled using the "surfaces" function in Imaris and classified based on gene expression using the filter tool. For each animal we calculated cell number in the following categories: 1) total *Esr1*, 2) total *Tac1*, 3) total *Rprm*, 4) total *Pdyn*, 5) *Tac1*/*Esr1*, 6) *Rprm*/*Esr1*, *and* 7) *Pdyn*/*Esr1*. Because *Esr1* was labeled in both series of sections, the mean for the first and second series was calculated for each animal and used in statistical analyses. Cells were counted in one or two matched sections containing the largest cross-sectional areas of the caudo-lateral VMHvl; if two sections were counted, an average was calculated and used in statistical analyses.

4.2.4 Statistics

GraphPad Prism was used for all statistical analyses. Two-way ANOVAs were used to examine effects of age and sex on each group of classified cells. Planned comparisons were evaluated using Fisher's Least Significant Difference following a significant main effect or interaction.

4.3 Results

4.3.1 Esr1 neuron number is high in both sexes at birth, but decreases in males at weaning

Esr1 gene expression mirrored the previously observed developmental pattern for ERα protein based on immunoreactivity (Mosley, Weathington et al., 2017). Namely, males and females had similarly high numbers of *Esr1* cells in the VMHvl at P1, but females had significantly more *Esr1* cells than males at P25 ($P < 0.0001$), due to a decrease in males from P1 to P25 ($P < 0.0001$) (Figure 4-1). This was reflected in significant effects of age ($F_{1,23} = 5.114$, $P = 0.03$) and sex ($F_{1,23} = 6.873$, $P = 0.015$), and an interaction between age and sex ($F_{1,23} = 16.98$, $P = 0.0004$) on *Esr1* cell number in the two-way ANOVA.

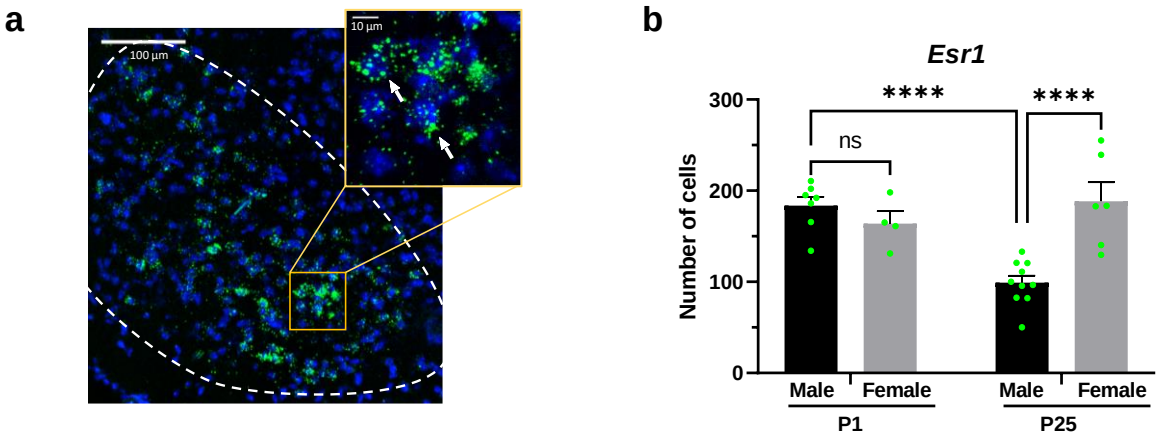

Figure 4-1 Sex-specific decrease in Esr1 neurons across development. *(A) Image depicts an outline of the VMHvl with nuclei stained with DAPI (blue) and Esr1 in green. Insert depicts a higher magnification view, with individual Esr1 cells indicated by arrows. (B) At P1, males and females have similar, high numbers of Esr1-expressing cells. Esr1 cell number subsequently decreases in males and females have a greater number of Esr1-expressing cells at P25. Mean + standard error of the mean and individual data points are depicted. ****P < 0.0001.*

4.3.2 Total Tac1 and Rprm neuron numbers are high in both sexes at birth, but decrease in males at weaning.

We found similar patterns for the total number of *Tac1* and *Rprm* cells: high numbers in both sexes at P1 but a decrease in males by weaning. For *Tac1*, we found a main effect of sex ($F_{1,18} = 5.12$, $P < 0.036$), with more *Tac1* cells in females (Figure 4-2). Although the age-by-sex interaction was not significant, we note that males had fewer *Tac1* cells than females at P25 ($P < 0.013$), and the total number of *Tac1* cells in males, but not females, decreased significantly between P1 and P25 ($P < 0.05$). For *Rprm*-expressing cells, we found main effects of age ($F_{1,18} = 45.56$, $P < 0.0001$) and sex ($F_{1,18} = 4.902$, $P = 0.04$), and an interaction between age and sex ($F_{1,18} = 27.20$, $P < 0.0001$). There was no sex difference in the number of *Rprm* cells at P1.

Rprm-expressing cells decreased between P1 and P25 only in males ($P < 0.0001$), resulting in a sex difference at weaning ($P < 0.0001$).

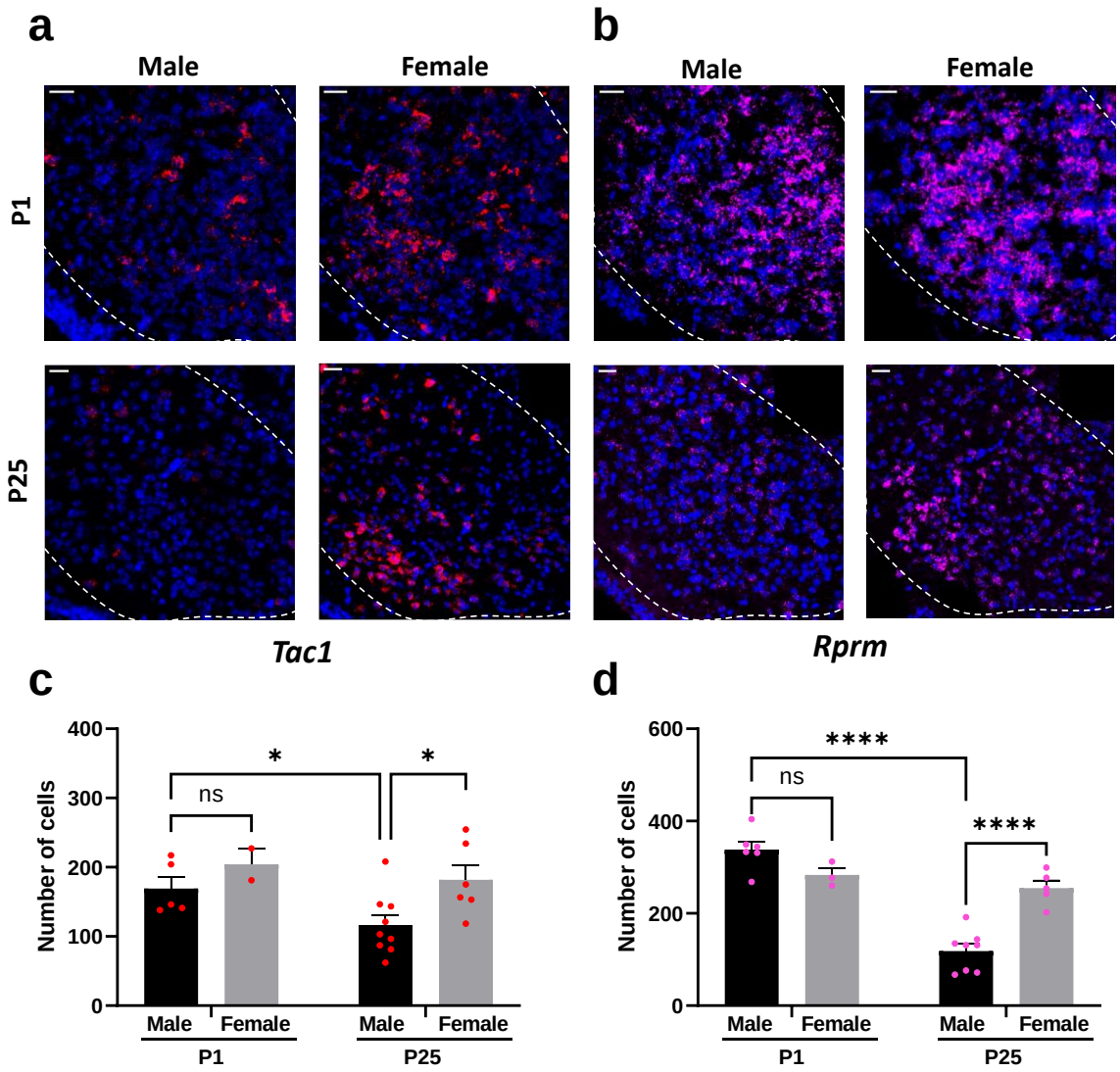

Figure 4-2 Sex-specific decreases in Tac1- and Rprm-expressing neurons across development. *Images depict Tac1 (A) and Rprm (B) expression in males and females at P1 and P25. Sex differences are not seen at P1 for either Tac1 (C) or Rprm (D). There is a decrease in Tac1 and Rprm cell number in males from P1 to P25, resulting in sex differences in both cell types at weaning. Mean + standard error of the mean and individual data points are depicted. Scale bars = 30 µm. *P < 0.05; ****P < 0.0001.*

4.3.3 A large sex difference in Pdyn neuron number is present at birth

A strikingly different pattern was seen for *Pdyn* neuron number in the VMHvl, which exhibited a marked sex difference in expression at birth, with 15-fold more *Pdyn* cells in males than in females on P1. However, similar to *Tac1* and *Rprm*, the number of *Pdyn* cells decreased in males ($P < 0.0007$) from P1 to P25, with no change in females (Figure 4-3). In the ANOVA, we found a main effect of sex ($F_{1,18} = 42.93$, $P < 0.0001$), and an interaction between age and sex ($F_{1,18} = 21.57$, $P = 0.006$). Males had more *Pdyn* cells than females at both ages (P1, $P < 0.0001$; P25, $P < 0.0045$), but the sex difference decreased between birth and weaning.

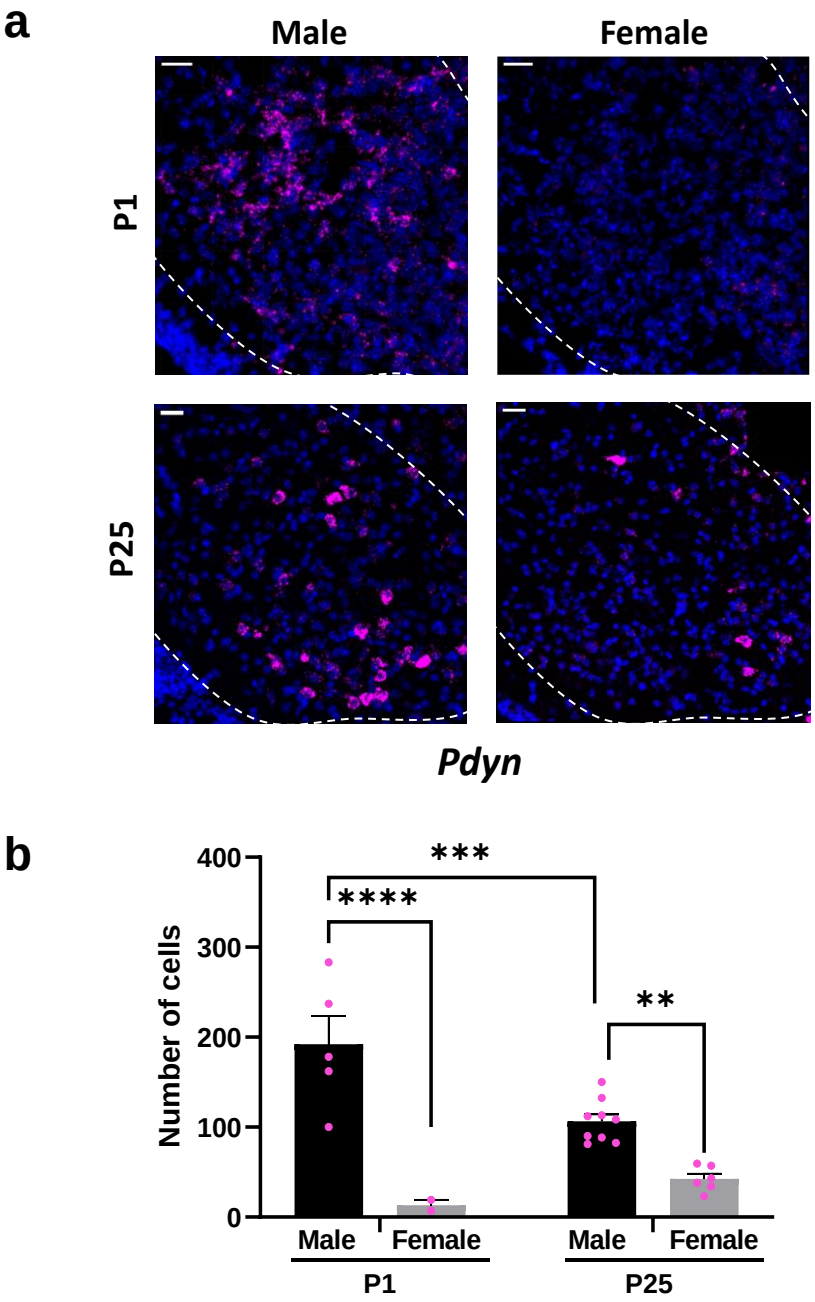

Figure 4-3 Males have more Pdyn cells than females at both ages*. (A) Photomicrograph depicting Pdyn expression in both sexes at P1 and P25. (B) Males have many more Pdyn cells than females at P1. There is a decrease in Pdyn cells in males from P1 to P25 but a sex difference remains at weaning. Scales = 30 µm. **P < 0.01; ***P < 0.001; ****P < 0.0001.*

4.3.4 Developmental changes in Esr1 cells co-expressing Tac1, Rprm, or Pdyn

To investigate developmental changes in ERα sub-populations, we calculated the number of *Esr1*+ cells in the VMHvl co-expressing *Tac1*, *Rprm*, or *Pdyn* at P1 and P25. For *Tac1*/*Esr1* cell number, we found main effects of age ($F_{1,18} = 8.37$, $P = 0.0097$), sex ($F_{1,12} = 21.23$, $P = 0.0002$), and a near significant interaction ($F_{1,18} = 3.5$, $P = 0.078$) (see Figure 4-4b). There was no sex difference at P1, but *Tac1*/*Esr1* cells decreased in males between P1 and weaning ($P = 0.0005$) and females had more *Tac1*/*Esr1* cells than males at P25 ($P < 0.0001$).

For *Rprm*/*Esr1* cells, we again found main effects of age ($F_{1,18} = 19.12$, $P = 0.0004$) and sex ($F_{1,18} = 12.54$, $P = 0.0023$), as well as a significant interaction ($F_{1,18} = 23.53$, $P = 0.0001$ (Figure 4-4c) due to a decrease in *Rprm*/*Esr1* cells only in males between P1 and P25 ($P < 0.0001$). This led to a sex difference at weaning age ($P < 0.0001$) that was not observed at birth.

For *Pdyn*/*Esr1* co-expressing cells, two-way ANOVA revealed significant effects of age ($F_{1,18} = 10.57$, $P = 0.0044$) and sex ($F_{1,18} = 38.33$, $P < 0.0001$), and an age-by-sex interaction ($F_{1,18} = 14.35$, $P = 0.0013$) (Figure 4-4d). There was a marked, 32-fold sex difference, favoring males, in *Pdyn*-expressing *Esr1* neurons at P1 ($P < 0.0001$) and a subtler, 4-fold sex difference at P25 ($P < 0.037$), resulting from a development decrease in *Pdyn*/*Esr1* co-expressing cells in males ($P < 0.0001$) from P1 to P25.

Thus, decreases in *Esr1* neurons co-expressing *Tac1*, *Rprm*, and *Pdyn* were superimposed upon a general decrease in the total number of neurons expressing each of these markers in males, while there was no change in any of the cell types in females. We confirmed that the decreases in *Esr1*, *Tac1*, *Rprm*, and *Pdyn* in males between P1 and P25 were independent of a change in total cell number in VMHvl based on counts of DAPI-stained cells (932 ± 34.3 (P1) vs 850.9 ± 33.8 (P25); $P = 0.14$). Similarly, the sex differences observed at P25 were not due to a

sex difference in total cell number in the VMHvl (850.9 ± 33.8 (males) vs 865.9 ± 45.82 (females); $P > 0.8$). This suggests instead a striking reconfiguration of VMHvl cell phenotype in males, but not females, during the first few weeks of life.

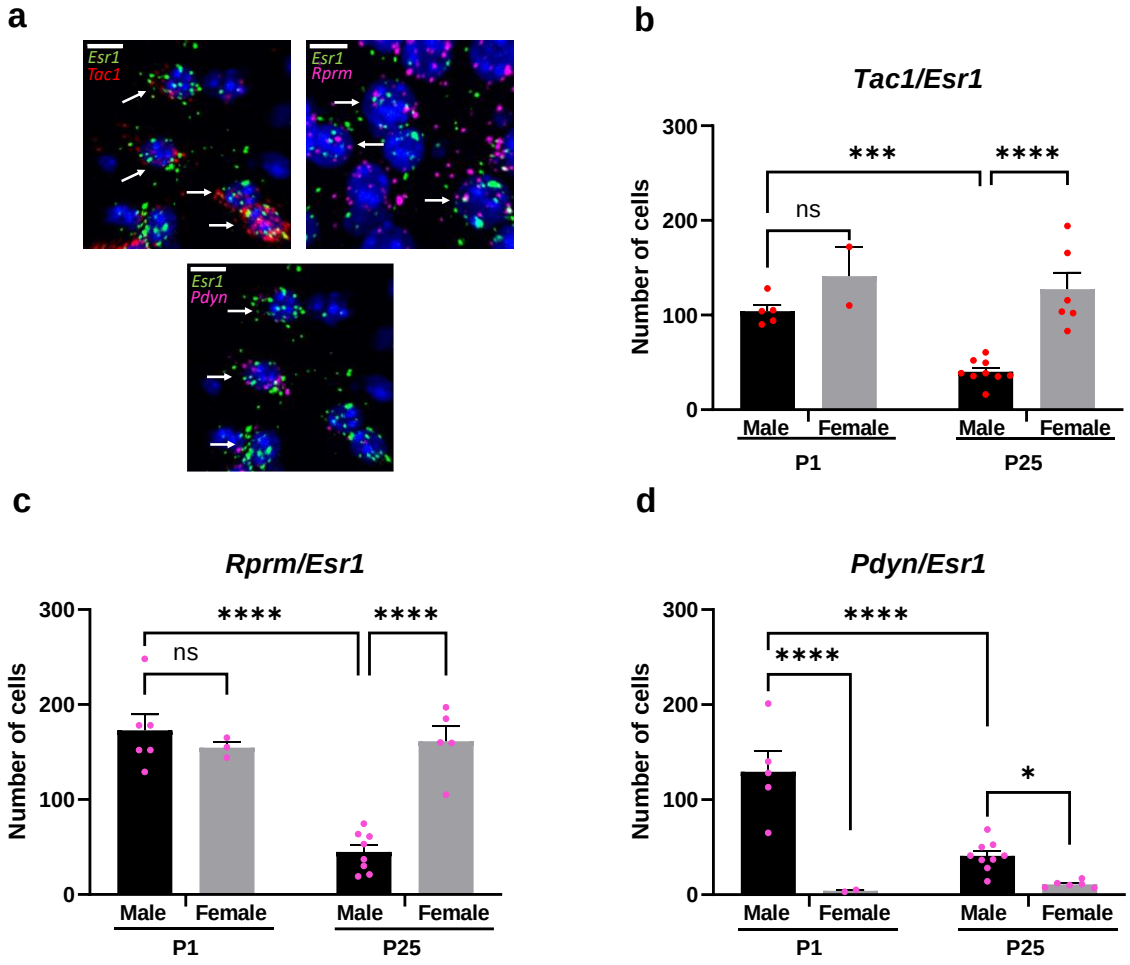

Figure 4-4 Esr1 co-expression with Tac1, Rprm, and Pdyn from birth to weaning. *(A) Photomicrographs depict cells (white arrows) expressing Esr1 with either Tac1 (top left), Rprm (top right), or Pdyn (bottom). (B) A sex difference in Tac1/Esr1 cells emerges at weaning, due a decrease in males, but not females. (C) Similarly, Rprm/Esr1 are high in both sexes at birth, but decrease in males only, leading to a sex difference at weaning. (C) Males have greater Pdyn/Esr1 counts at P1. There is a decrease in Pdyn cells in males from P1 to P25, but a sex difference remains at weaning. Scales = 10 µm. *P < 0.05; ***P < 0.001; ****P < 0.0001.*

4.4 Discussion

The present findings support our previous observations on the development of the sex difference in ERα protein in the VMHvl (Mosley, Weathington, et al., 2017). *Esr1* cell number in the VMHvl was equally high in both sexes at birth, and decreased over the next few weeks only in males, resulting in a sex difference at weaning. The sex difference in ERα is eliminated when mice are treated with a DNA methylation inhibitor at birth (Mosley, Weathington, et al., 2017), and a small, but significant, sex difference in methylation of the *Esr1* promoter region (males > females) is observed at weaning (Cortes et al., 2021). Thus, the number of cells expressing *Esr1* mRNA decreases in males during postnatal development, and this may be due to DNA methylation.

We also found that the total number of *Tac1*- and *Rprm*-expressing cells, and the number of *Tac1*/*Esr1* and *Rprm*/*Esr1* co-expressing cells in the VMHvl was similar between both sexes at birth. All four of these cell types decreased in males between birth and weaning, while remaining constant in females. Thus, sex differences were seen at weaning in *Tac1*, *Rprm*, and *Tac1/Esr1*, *Rprm/Esr1* cells, as was previously reported for adult mice (van Veen et al., 2020). The fact that these differences emerge prior to puberty supports the suggestion that they are independent of circulating gonadal steroid hormones.

In contrast to the female-bias in *Tac1* and *Rprm* cells in the VMHvl, males had 15 times more *Pdyn* cells as females at P1, and over 30 times as many *Pdyn*/*Esr1* co-expressing cells. Both cell types exhibited a developmental decrease in male, but not female mice, resulting in smaller sex differences at weaning (2.5-fold more *Pdyn* cells and 4-fold more *Pdyn*/*Esr1* cells in males at P25). Total cell number in the VMHvl did not decrease in males between P1 and P25 and was not different between males and females at P25, confirming that changes in cell

phenotype, rather than cell death, account for the developmental decreases in cells expressing these markers.

Our hypothesis that sub-populations of ERα cells implicated in female-specific physiological processes decrease in males, but not females, across development was partially supported by the observed decrease in *Tac1*/*Esr1* and *Rprm*/*Esr1* cell number. However, we also saw a decrease in *Pdyn*/*Esr1* cells in males, suggesting a general decrease across all subtypes of ERα neurons.

4.4.1 Roles of Tac1, Rprm, and Pdyn

Of the three genes co-expressed with *Esr1* in the VMHvl that we examined (*Tac1*, *Rprm*, *Pdyn*), the role of the *Tac1* product, substance P, has been the most extensively studied. *Tac1* mRNA can be spliced into four variants (α, β, γ-, and δ-TAC1) which all generate substance P (Joos & De Swert, 2006). β-Tac1 can also be processed into neurokinin A or neuropeptide κ, while γ-Tac1 can yield neuropeptide γ. Substance P immunoreactive cells in the VMHvl, project to the midbrain central gray (Dornan et al., 1990) to facilitate lordosis (Dornan et al., 1987). Although 50% of substance P cells in the VMHvl express ERα in rats (Akesson & Micevych, 1988), the role of SP/ERα cells, specifically, in lordosis has not been tested. In addition, a sub-group of VMHvl neurons that express ERα, *Tac1*, and *Nkx2-1* are implicated in estrogen-dependent spontaneous locomotion in females (Correa et al., 2015).

The product of the *Pdyn* gene, prodynorphin (also referred to as pro-enkephalin B or PENK-B), is a precursor protein that can be cleaved into various opioid peptides: dynorphin A, dynorphin B, α-neoendorphin, and β-neoendorphin. These opioids act as neuromodulators and hormones through binding of κ-opioid receptors (Kieffer, 1995; Fricker et al., 2020), although some have suggested an opioid-independent mechanism transiently active during the perinatal

period that involves NMDA receptors and prostaglandins (Szeto, 2003), both of which are found in the VMHvl (Kia et al., 2002; Szeto, 2003; M.-L. Lee et al., 2021). Research on the function of *Pdyn* in the VMHvl is sparse, although a role in control of the hypothalamic-pituitary-adrenal axis has been proposed (Khachaturian et al., 1985; Spampinato et al., 1988).

Rprm is a recently identified gene conserved across many species and expressed predominantly in the brain (Wichmann et al., 2016; Amigo et al., 2018; Stanic et al., 2018). Aside from the female-specific role of *Rprm/Esr1* cells in thermoregulation recently discovered by van Veen et al. (2020), not much is known about the function of *Rprm* in the brain. In mouse embryonic fibroblasts, DNA damage invokes P53-mediated induction of *Rprm*, and overexpression of *Rprm* leads to cell cycle arrest (Ohki et al., 2000). The loss of *Rprm* expression through hypermethylation of its promoter is also associated with cancer progression (Luo et al., 2011; Saavedra et al., 2015). Neurogenesis is almost exclusively prenatal in the hypothalamus (Shimada & Nakamura, 1973; Jacobson & Gorski, 1981; Bayer & Altman, 1987), yet we found very high expression of *Rprm* in the newborn VMHvl of both sexes, suggesting a potential developmental role unrelated to cell cycle regulation.

4.4.2 Sex differences in Tac1, Rprm, Pdyn

An earlier study in rats found no sex difference in substance P content in the VMH between diestrous females and males (Frankfurt et al., 1985). Micevych et al. (1988) found variations in substance P content in the VMH across the estrous cycle in rats, with a drop in estrous females that the authors attributed to increased peptide release (Micevych et al., 1988). However, we find a sizeable sex difference in *Tac1* cell number prior to puberty in mice, suggesting either technical (entire VMH vs VMHvl; protein vs mRNA) or species (rat vs mouse)

differences, or that there are both organizational and activation effects of hormones on *Tac1* levels.

In contrast to *Tac1* and *Rprm*, the sex difference in *Pdyn* in adult mice is eliminated by adult gonadectomy, and thus depends on activational (*i.e.*, transient) effects of gonadal hormones (van Veen et al., 2020). If *Pdyn* is acutely responsive to sex steroid hormones throughout life, it may explain the marked sex difference found at P1, when males, but not females, have a surge in circulating and hypothalamic sex steroid levels (Weisz & Ward, 1980; Rhoda et al., 1984; Corbier et al., 1992; Konkle & McCarthy, 2011). In contrast, effects of the perinatal hormone surge may program latent sex differences in *Tac1* and *Rprm*, which do not emerge until later. The developmental decrease in *Pdyn* cell number in males from P1 to P25 may be due to the very low levels of gonadal steroids after the neonatal surge (Selmanoff et al., 1977; Clarkson et al., 2012).

Based on radioimmunoassay, others have reported no sex differences at weaning in concentration of prodynorphin-derived peptides (*e.g.*, dynorphin) in the rat hypothalamus (Seizinger et al., 1984); although they also reported a developmental pattern opposite to that observed here with increasing levels of dynorphin concentration from birth to adulthood. The discrepancy between those findings and ours may be due to species differences (rats vs mice), difference between protein and mRNA, or dissection method: they extracted a large portion of the hypothalamus, whereas our data pertain specifically to the VMHvl.

4.4.3 Overview

The sex differences in *Tac1*, *Rprm*, *Pdyn*, and *Esr1* cell number observed at weaning are likely due to the organizational effects of testosterone, and I am currently testing whether neonatal testosterone propriate exposure is sufficient to masculinize expression of these markers

in the VMHvl of female mice. Most effects of testosterone in sexual differentiation of the rodent brain are mediated by ERα after the aromatization to estradiol (Rissman et al., 1997; Ogawa et al., 2000; Bodo et al., 2006; Wu et al., 2009; Juntti et al., 2010). In support of this, *Esr1* genetic ablation in the VMHvl completely feminizes *Rprm* and *Tac1* expression (van Veen et al., 2020). We posit that sexual differentiation of *Tac1*, *Pdyn*, and *Rprm* is due to testosterone acting via ERα to permanently downregulate expression.

ERα could influence *Tac1* expression by binding to the estrogen response element found upstream of its promoter region (Bourdeau et al., 2004) and/or through epigenetic mechanisms. Hypermethylation of *Tac1* is associated with gene silencing, at least in colon cells during cancer progression (Mori et al., 2006). In breast cancer cells, *Rprm i*s a direct target of ERα and is repressed at physiological doses of estradiol (Malik et al., 2010). Estradiol-mediated repression of *Rprm* requires ERα and a histone deacetylase, and is associated with decreased levels of histone methylation in the *Rprm* promoter region (Malik et al., 2010). Future studies could manipulate epigenetic processes to determine the downstream mechanisms by which estradiol/ERα orchestrates sexual differentiation of sub-populations of ERα neurons.

In summary, we found no sex differences in *Esr1, Tac1, Rprm, Tac1*/*Esr1, or Rprm*/*Esr1* cell number at birth; sex differences favoring females emerged by weaning, and in all cases were due to down regulation in males. The number of *Pdyn* and *Pdyn*/*Esr1* co-expressing cells, although greater in males at both ages studied, also decreased from birth to weaning in males. This suggests considerable reorganization of the VMHvl in males during postnatal development, likely due to phenotypic specification organized by ERα and the perinatal peak in gonadal steroids. This may orchestrate functional differences in a key hypothalamic hub controlling reproduction and homeostasis in preparation for puberty.

5 GENERAL DISCUSSION

5.1 Overview of sex differences in the nervous system

The existence of sex differences in the brain has long been speculated. In 300 BC, Aristotle noted that women have smaller brains than men (Mayhew, 2004), and many of the first observations of sex differences in the brain were similarly gross in description (Hamlin, 2014). More recently, many more subtle sex differences have been described that presumably underlie differences in physiology and behavior in males and females. Understanding the biological underpinnings of neural sex differences is imperative to gain mechanistic insight into neurodevelopment and sex-biased neuropsychiatric disorders.

In the mid to late 1900's, technological advances and a burgeoning interest in the brain led to the first discoveries of structural differences in male and female brains. Raisman & Field (1973) uncovered sex differences in dendritic morphology using electron microscopy, while Gorski et al. (1978;1980) found a dramatic morphological difference in the size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in rats, which was later extended to humans by Swaab & Fliers (1985). A sex difference in the number of motoneurons in the spinal nucleus of bulbocavernosus (SNB) which innervates the perineal muscles was discovered in the rat by Breedlove & Arnold (1980), and in the homologous Onuf's nucleus in humans and dogs by Forger & Breedlove (1986).

In the decades following, there was intense interest in uncovering the mechanisms causing these sex differences in nuclear volume or neuron number. In principle, a sex difference in any of four major developmental processes could be responsible: 1) neurogenesis, 2) neuronal migration, 3) neuronal cell death, and 4) phenotypic specification (for reviews, see Forger, 2006; Forger 2009). However, the generation and migration of most neurons are complete prior to

birth, *i.e*., before the surge in testicular hormones (Weisz & Ward, 1980) that has been linked to many of these sex differences. Neurogenesis has been largely ruled out as a major contributor to neural sex differences (Jacobson & Gorski, 1981; Park et al., 1996; Henderson et al., 1999), and there is limited, somewhat contradictory evidence for a role of cell migration (Jacobson et al., 1985; Park et al., 1996; Henderson et al., 1999).

On the other hand, developmental neuronal cell death coincides with the critical period of sexual differentiation of the brain (Ahern et al., 2013), and has been successfully attributed to the development of several well-known structural sex differences (Ito et al., 1986; del Abril et al., 1987; Arai et al., 1994). Sex steroid hormones alter developmental cell death in a region-specific manner resulting in differing nuclear volumes between males and females. For example, testosterone is neuroprotective in the SDN-POA, SNB, and the bed nucleus of the stria terminalis (BNST), and thus females have a greater number of dying cells in the perinatal period resulting in smaller volumes and cell number in these regions in adulthood (Gorski et al., 1978; Breedlove & Arnold, 1980; Nordeen et al., 1985; del Abril et al., 1987; Davis et al., 1996; Chung et al., 2000; Kato et al., 2012).

In contrast, the anteroventral periventricular nucleus (AVPV) has many more neurons in females (Sumida et al., 1993). Here, testosterone increases cell death resulting in a smaller nucleus in males (Nishizuka et al., 1993; Arai et al., 1994). Testosterone may influence critical cell death regulators, as male rats have greater expression of the pro-apoptotic gene *Bax*, and lower levels of the apoptotic suppressor *Bcl2* in AVPV-containing tissue at birth (Tsukahara et al., 2006). Deletion of *Bax* or over-expression of *Bcl2* eliminates the sex differences in AVPV, SNB and BNSTp cell number (Zup et al., 2003; Forger et al., 2004).

However, there is another type of sex difference that remains in mice lacking *Bax* or over-expressing *Bcl2.* These mice still have neurochemical sex differences in the number of neurons expressing tyrosine hydroxylase or kisspeptin in the AVPV, for example (Zup et al., 2003; Forger et al., 2004; Semaan et al., 2010). Likewise, sex differences in vasopressin number in the BNSTp (de Vries et al., 2008) and calbindin number in the SDN-POA (Gilmore et al., 2012) remain in *Bax* knock-out mice.

These remaining sex differences fall under the umbrella of differences in *cell phenotype*, which is defined by stable patterns of gene expression. This type of sex difference, although more subtle than the differences in nuclear volume, are likely common throughout the brain and body. Cell phenotype differences are also likely to have broad consequences, as differences in gene expression underlie differences in neural function, as well as differences in morphology, connectivity, and neurotransmitter/neurosteroid production. Epigenetic mechanisms orchestrate cell fate differentiation during development (Hsieh & Gage, 2004; Iqbal et al., 2012), and likely play an important role in specifying neural phenotypic decisions.

5.2 Overview on epigenetics and structural and behavioral sex differences

The first study to test the role of epigenetics in sexual differentiation of the brain manipulated histone modifications. Murray and colleagues (2009) noted a delay between the neonatal testosterone surge and sexually dimorphic cell death, and thus hypothesized that epigenetic mechanisms may underlie the cellular memory for neonatal steroid hormone exposure. Given that steroid hormones often recruit histone acetyltransferases (M. Y. Kim et al., 2001; Welboren et al., 2009), they predicted and discovered that neonatal histone de-acetyltransferase inhibition prevents masculinization of BNSTp volume in mice (Murray et al., 2009). Later, HDAC inhibition was discovered to reduce, but not eliminate, the sex difference in

vasopressin immunoreactivity in the lateral septum and masculinize olfactory preference for female-soiled bedding in female mice (Murray et al., 2011).

Interestingly, many sex differences, including those of neurochemical phenotype, are latent (*i.e.*, are established early in life, but do not appear until later). For example, sex differences in ERα emerge several days or even weeks after the neonatal testosterone surge (Yokosuka et al., 1997; Kelly et al., 2013). This may involve the placement of epigenetic marks, such as histone modifications and/or DNA methylation, capable of maintaining the "memory" of steroid hormones long after exposure. We focused on DNA methylation in this dissertation because it is considered the most stable epigenetic mark. In fact, DNA methylation was considered a permanent mark until the discovery of ten eleven translocases (TETs) by Tahiliani and colleagues (2009), which provided a mechanism for active DNA demethylation.

Two independent groups described sex differences in levels of DNA methylation in the promoter regions of the rat *Esr1* in the POA (Schwarz et al., 2010; Kurian et al., 2010). Moreover, the sex differences in DNA methylation were eliminated in females administered estradiol early in life, suggesting that gonadal hormones leave an epigenetic imprint. Later, Nugent and colleagues (2015) discovered sex differences in the catalytic activity of DNA methyl transferase enzymes (DNMTs) in the rat POA (female > male), accompanied by sex differences in global methylation levels (female > male; Nugent et al., 2015). Both sex differences in DNMT activity and methylation levels were eliminated by neonatal administration of estradiol to females. Injecting a DNMT inhibitor (zebularine) into the cerebral ventricles of neonatal rats masculinized POA dendritic morphology and sexual behavior in female rats (Nugent et al, 2015). The authors concluded that feminization of the brain may not be the passive, "default" process once believed, but in fact, entails active repression via DNA methylation. The findings described

in this dissertation suggests a more nuanced role for DNA methylation in sexual differentiation, at least in the mouse brain.

5.3 First study on the role of DNA methylation in sexual differentiation of neurochemical phenotype

We were interested in whether DNA methylation underlies three sex differences in neurochemical phenotype: the number of calbindin-expressing neurons in the SDN-POA and the number of ERα-expressing neurons in the POA and VMH. To test this, we intracerebroventricularly injected zebularine into the brains of male and female mice on the first two days of life, and examined their brains at weaning. Neonatal DNMT inhibition abolished sex differences in ERα cell density in the VMHvl and mPOA by increasing the number of ERα cells in *male* mice at weaning (Mosley, Weathington et al., 2017). In the same animals, the sex difference in calbindin cell number was decreased by increasing the number of calbindin cells in *females*. Thus, our findings suggested that DNA methylation is capable of both feminization and masculinization, depending on the endpoint observed.

Neonatal zebularine treatment did not alter developmental cell death in the mPOA and VMHvl, or total cell density in the mPOA, suggesting that rather than affecting cell survival, zebularine impacted the proportion of cells able to express these markers. We also found that both sexes had high levels of ERα at birth, which was unaffected by zebularine 6 hours after the last injection (Mosley, Weathington et al., 2017), confirming that sex differences in ERα are latent, and that zebularine has long-term effects on expression. This study was the foundation of the first aim of my dissertation, where I sought to examine the role of DNA methylation in hormone-mediated sexual differentiation of ERα, kisspeptin, and calbindin cells in the hypothalamus.

5.4 Chapter 2: Neonatal inhibition of DNA methylation disrupts testosterone-dependent masculinization of neurochemical phenotype

Sex differences in calbindin, ERα, and kisspeptin are present prior to puberty (Yokosuka et al., 1997; Sickel & McCarthy, 2000; Clarkson & Herbison, 2006; Kauffman et al., 2009) suggesting a role for either sex chromosome complement or organizational effects of gonadal hormones. We hypothesized that perinatal exposure to sex steroid hormones may impart long-term influences on expression of these markers through DNA methylation. If so, we predicted that testosterone-mediated masculinization would be impaired in animals treated neonatally with a DNMT inhibitor.

Indeed, we found that females treated with testosterone propionate during the first two days of life had complete masculinization of ERα in the VMHvl and mPOA, kisspeptin in the AVPV/periventricular nucleus (PeN), and calbindin in the mPOA and BNSTp. Neonatal zebularine treatment partially prevented hormone-dependent masculinization: females treated with testosterone and zebularine had a higher (*i.e.*, more control female-like) number of ERα-immunoreactive cells in the VMHvl, than females administered testosterone alone. Intriguingly, we found that the sex difference in kisspeptin in the AVPV/PeN was unaffected by zebularine treatment, and that testosterone-mediated masculinization was also not altered by DNMT inhibition. Both testosterone and zebularine increased (*i.e.*, masculinized) expression of calbindin in the POA, which did not allow us to disentangle the actions of testosterone from DNA methylation. However, we found that calbindin cell number was increased only in females by zebularine treatment, eliminating group differences between females, males, and masculinized females in the SDN-POA. A similar female-specific increase after neonatal inhibition of DNA methylation was found for calbindin cell number in the BNSTp.

These findings suggest that DNA methylation regulates sex differences in neurochemical phenotype in a cell-type or region-specific fashion. We did not fully prevent masculinization of ERα in the VMHvl with zebularine, and that could be due to a combination of incomplete inhibition of DNMTs by zebularine (indeed, we found a 50% reduction in DNMT activity following treatment) or supraphysiological levels of testosterone, or simply the existence of several, non-overlapping mechanisms by which testosterone may have long-term effects on ERα levels.

We used testosterone to masculinize females in this study, to mimic the normal situation encountered by neonatal males. However, most aspects of sexual differentiation in rodents depend on the conversion of testosterone to estradiol within the brain (Bakker et al., 2003). A limitation of our work is that we are unable to tease apart the effects of estrogenic metabolites, which act on ERs, from testosterone itself or the androgenic metabolite, dihydrotestosterone, which act on androgen receptors. Nugent et al. (2015), Schwarz et al. (2010), and Kurian et al. (2010) report changes in global or *Esr1* promoter methylation in hypothalamic tissue after neonatal treatment with estradiol benzoate. Furthermore, treatment with estradiol, but not dihydrotestosterone, alters DNA methylation levels *in vitro* in repetitive gene elements (LINE and Alu elements; a proxy for global methylation), only in breast epithelial cells that express estrogen receptor (El-Maarri et al., 2011). I speculate that the masculinizing effects of testosterone that we observed depend on estrogen receptors and act on the epigenome to establish long-term sex differences in ERα expression and likely other facets of sexual differentiation of the brain.

An important question that remains is *how* might the effects of sex steroid hormones and epigenetic processes intersect to shape the brain? If testosterone/estradiol does indeed influence

gene expression through DNA methylation, what is the mechanism? Estradiol does not seem to affect the expression of *Dnmt1*, *Dnmt3a,* or *Dnmt3b* in the postnatal POA, but does decrease the catalytic activity of DNMTs up to three days after treatment at birth in rats (Nugent et al., 2015). *Dnmt3L* is another gene in the DNMT family that does not have its own catalytic domain, but increases the efficiency of DNMT3a (Chédin et al., 2002). Both *Dnmt3a* and *Dnmt3L* have conserved estrogen response elements, regulatory sites by ligand-bound ERα in the mouse and human genome (Bourdeau et al., 2004). Thus, I speculated that gonadal steroid exposure early in life may decrease expression of *Dnmt3L,* which may then decrease the catalytic activity of DNMT3a and underlie sex differences in epigenetic processes. The effect of neonatal sex steroid hormones on *Dnmt3L* expression in the hypothalamus was tested in following section.

5.5 Sex differences in DNA methylation and demethylation in the postnatal hypothalamus

In work that I participated in, but that does not form part of this dissertation, we found peak expression of the enzymes that add and remove DNA methylation marks (DNMTs, TETs) during the first postnatal week in the mouse brain (Cisternas, Cortes, Bruggeman et al., 2020). Similar to Nugent et al. (2015), we also found greater global methylation levels in the POA in females relative to males, but only at P7. However, testosterone treatment of females did not reduce the sex difference in global methylation, but rather amplified it (females + T > female + vehicle).

Females had higher expression of *Dnmt1* and *Dnmt3L* in the POA at P7, in contrast to the lack of sex differences in DNMT expression in the neonatal rat (Nugent et al., 2015). We also found that expression of *Dnmt3L* was low at birth, but increased steadily to P60 – showing an

opposite pattern to the other DNMTs and TETs which peaked in the first week and steadily decreased.

No previous study had examined hydroxymethlation in relation to sexual differentiation. We did not observe sex differences in global hydroxymethylation levels in the POA, VMH, or hippocampus (Cisternas, Cortes, Bruggeman et al., 2020). However, males had higher expression of TETs in the VMH and POA at P1 or P7. This suggested that DNA hydroxymethylation may play an important role in sexual differentiation of the brain, and this idea was tested in Chapter 3.

5.6 Chapter 3: DNA methylation and demethylation underlie sex differences in estrogen receptor alpha in the arcuate nucleus

In examining the tissue generated for Chapter 2, we noted that female mice had greater labeling of ERα not only in the VMHvl, but also in the neighboring arcuate nucleus (ARC) (Cortes et al., 2021). This sex difference in ERα labeling was not seen at birth, but was present at weaning, as was previously seen for the sex difference in the VMHvl (Mosley, Weathington et al., 2017). We also determined that neonatal zebularine treatment abolished the sex difference in ERα in the ARC. However, here we found that zebularine masculinized (*i.e.*, reduced) labeling of ERα in the ARC of females – in contrast to the effect found in the VMHvl where zebularine had feminized (*i.e.*, increased) ERα in males. Zebularine treatment did not alter cell death in neonates, nor total cell number at weaning in the ARC, confirming that we disrupted phenotypic differentiation, and not cell survival.

Because we found greater *Tet2* and *Tet3* enzyme expression in the hypothalamus of males during the first week of life (Cisternas, Cortes, Bruggeman et al. 2020), we down-regulated expression of these enzymes in neonatal males using siRNA to test their role in sexual differentiation of ERα. We found that males treated with siRNA against *Tet2* and *Tet3* had

feminized (*i.e*., increased) labeling of ERα in the ARC at weaning. In combination with the zebularine results, our data suggested that inhibiting DNA methylation decreases gene expression of *Esr1* in the ARC, while inhibiting demethylation increases it. In the VMHvl, there was no effect of siRNA against these TETs suggesting regional differences in regulation of *Esr1* cells.

We hypothesized that sex differences in ERα in the hypothalamus are associated with sex differences in the methylation status of the *Esr1* promoter. Because there are no sex differences in ERα labeling at birth in the ARC or VMHvl, we predicted that sex differences in methylation of the *Esr1* promoter would not emerge until weaning. Indeed, we found that males and females had similar percent levels of methylation at CpG sites in a key *Esr1* promoter region in both the ARC and the VMHvl at P1. At weaning, however, we found interesting region-specific sex differences in methylation: males had greater *Esr1* methylation in the VMHvl (as predicted if methyl marks are repressive), while females had greater *Esr1*methylation in the ARC (as predicted if methyl marks are activating). We note that the sex differences were subtle (~2% in absolute value), although they were in the same direction for every CpG site examined.

Overall, the average percent methylation of the *Esr1* promoter regions we examined was relatively low (~3 – 20%, depending on region and CpG site). This was surprising since we assayed punches of the ARC and VMHvl, which contain many cells that do not express *Esr1* (and that we would therefore expect might have high methylation of the *Esr1* promoter). The *Esr1* methylation levels we observed at P1 were similar to levels previously observed at the same CpG sites at P4 in the isocortex (Prewitt & Wilson, 2007; Westberry et al., 2010). Nonetheless, the overall low methylation status of our tissue suggests that regulation of ERα expression may also occur at other genomic regions and/or through parallel mechanisms. For example, steroid

hormones regulate gene expression through changes in chromatin structure mediated by co-activators with histone acetyltransferase (HAT) activity, such as the steroid receptor coactivator family of proteins and P300/CREB binding protein (M. Y. Kim et al., 2001). Future studies could use HAT inhibitors to test the role of histone acetylation in regulation of ERα and other steroid responsive genes.

Because neonatal zebularine treatment caused an increase in ERα cell number in the VMHvl of males, we predicted that promoter methylation of *Esr1* in VMHvl punches would be reduced in zebularine-treated males. Unexpectedly, we found no effect of zebularine on methylation levels of the promoter regions we examined. Also surprising, we found that zebularine-treated female mice had slightly higher levels of methylation in the ARC than controls. In peripheral cells, a DNA methylation inhibitor (5-azacytidine) previously caused downregulation of gene expression through demethylation of *gene bodies* (Yang et al., 2014). Future analyses should widen the genomic area examined to encompass transcribing regions, especially for genes such as *Esr1* in the ARC that show an unexpected decrease in expression after inhibition of DNA methylation. Another limitation of our work is that we only examined two points in development, the first point prior to the sex difference and zebularine effect on ERα, and the second at which they are both observed. It is possible we missed a window during which zebularine had the predicted effect on *Esr1* promoter methylation. Related, it is possible that our second "snapshot" of *Esr1* promoter methylation reflected compensatory epigenetic marks due to effects of zebularine elsewhere. Future studies could examine the ontogeny of sex differences in DNA methylation levels of the *Esr1* promoter (and its gene body), which would also help pinpoint *when* epigenetic editing should be used *in vivo* to test the causality of these marks in regulating *Esr1* gene expression (another major limitation of our study).

Finally, the design of our pyrosequencing study did not allow us to distinguish between mC and hmC, as sodium bisulfite treatment only distinguishes unmodified cytosines from modified cytosines. We observed that female mice have greater methylation levels in the *Esr1* promoter at weaning relative to males and assume it is mC, since hmC is enriched within the gene body in cerebellar tissue, as opposed to promoters (Szulwach et al., 2011). However, hmC is associated with increased gene expression and could explain our findings instead of a possible non-canonical association of mC with promoting gene expression. In the future, MeDIP and hMeDIP could be used to resolve the DNA methylation marks that are sexually differentiated in both the VMHvl and ARC.

5.7 Sexual differentiation of ERα subpopulations in the VMHvl

In regions such as the VMHvl, ERα-expressing neurons are not a homogenous cell group, but are comprised of multiple subtypes, with various projections and functions. Given the crucial role of neurochemistry in neuron function, the "decision" of a cell to express or not express a given receptor (*e.g.,* ERα) and/or certain neuropeptides (*e.g.*, substance P, prodynorphin,) has functional ramifications for the entire neural circuit, and consequently, down-stream behavioral and physiological processes.

Both sexes start out with an equally high number of ERα neurons in the VMHvl at birth, based on immunohistochemical detection of ERα cells (Mosley, Weathington et al., 2017)**.** Here, we extended this observation to the mRNA level (*Esr1*) and discovered that newborns of both sexes also have high numbers of *Tac1* and *Rprm* cells, as well as cells that co-express *Tac1* or *Rprm* with *Esr1* (*Tac1*/*Esr1* and *Rprm*/*Esr1*). In contrast, there is a marked sex difference in *Pdyn* cells and *Pdyn/Esr1* co-expressing cells, favoring males, at birth. All of the cell types we examined decreased from birth to weaning in males, while remaining unchanged in females

(*Esr1*, *Tac1*, *Rprm*, *Pdyn*, *Tac1*/*Esr1*, *Rprm*/*Esr1*, *Pdyn*/*Esr1*). Our results demonstrate that sex differences previously found in the VMHvl of adult mice (van Veen et al., 2020) develop between birth and weaning and do not require a post-pubertal, adult-like hormonal milieu. As van Veen et al. (2020) had already ruled out a role for sex chromosome complement, this strongly supports the proposal that these sex differences are established through the programming action of sex steroid hormones early in life.

As mentioned above, most actions of testosterone on sexual differentiation of the rodent brain occur through ERα, and *Tac1*, *Rprm* and *Pdyn* all have high colocalization with ERα early in life. Thus, we posit that the male-specific decreases of *Tac1*, *Rprm*, and *Pdyn* depend on the actions of ligand-bound ERα in the neonatal period. Indeed, genetically ablating *Esr1* in VMHvl cells increases *Tac1* and *Rprm* expression levels in males, while having no effect in females (van Veen et al., 2020). However, it is unknown whether *Esr1* deletion alters a phenotypic "decision" (*i.e.*, the number of cells fated to express *Tac1* and *Rprm*), as we might expect based on our findings, or simply the levels of mRNA produced per cell.

We speculate that the cellular composition observed in males and females at birth may be the "default" which, in the absence of sex steroid hormones early in life, leads to the development of neural circuitry that allows for estrogenic regulation of homeostatic processes and behavior in adulthood. In males, this circuitry is abolished in response to perinatal gonadal hormones, resulting in fewer estrogen-responsive *Tac1* and *Rprm* cells and subsequent inability for the animal to readily display lordosis or estrogen-dependent changes in locomotion and thermoregulation. I predict that females treated with testosterone perinatally would have masculinized (*i.e.*, reduced) expression of *Tac1*, *Rprm*, and *Pdyn*, as well as *Tac1/Esr1*, *Rprm/Esr1*, and *Pdyn/Esr1*, and am currently conducting an experiment to test this. If so, it

would also be of interest to test if there are functional consequences associated with the elimination of those cell types (such as decreases in spontaneous physical activity or body temperature; Correa et al., 2015; van Veen et al., 2020).

5.8 Working hypothesis, limitations, future directions

My dissertation adds to the body of literature demonstrating a role for epigenetics in sexual differentiation of the brain and, to our knowledge, contributes the first studies to demonstrate a role of DNA methylation in establishing sex differences in neurochemical phenotype. We find heterogeneity in the regulation of neurochemical sex differences, even within the same brain region. While sex differences in ERα, calbindin, and kisspeptin are all established by exposure to gonadal hormones early in life, inhibition of DNA methylation only affects the expression of some cell types in a region-specific manner, demonstrating that gonadal hormones exert their influence on gene expression through multiple independent pathways.

Neonatal inhibition of DNA methylation has region-specific effects on ERα (VMHvl vs ARC), suggesting that even the same cell type may be differentially regulated depending on its location and/or co-expression with other neuropeptides and regulatory proteins. We hypothesize that DNA methylation acts canonically to dampen *Esr1* gene expression in the VMHvl, while instead correlating with increased *Esr1* expression in the ARC. *Esr1* is not the only gene whose expression is down-regulated in VMHvl cells across development: we observe that *Tac1*, *Rprm*, and *Pdyn* cell number, with and without *Esr1* co-expression, decreases from birth to weaning. I speculate that exposure to gonadal steroids early in life causes ERα to modulate its own expression in a process that at least partly depends on DNA methylation, and this in turn alters the ability of cells to express functionally relevant peptides and proteins.

If epigenetic enzymes help shape sexual differentiation of the brain, how are specific genes targeted? In other words, what confers specificity to TETs and DNMTs? While the answer to this is largely unknown, recent studies suggests that TET2 is a part of the ERα signaling complex in breast cancer cells and is necessary for proper ER binding to DNA (Wang et al., 2018; Broome et al., 2021). ERα and TET2 co-occupy regulatory sites across the genome, and their cooperation is necessary for proper expression of ERα-regulated genes (Broome et al., 2021). Furthermore, the *Tet2* gene has ERα bindings sites (Broome et al., 2021) and *Tet2* expression is induced by estradiol in breast cancer cells (Wang et al., 2018).

This may extend to the brain, as we saw greater *Tet2* expression in in the hypothalamus of males during the critical period of sexual differentiation when males have higher gonadal hormone levels (Cisternas, Cortes, Bruggeman et al., 2020). While some genes, such as *Esr1* in the VMHvl, may have more mC marks in males than females, the female hypothalamus has overall greater global levels of methylation, at least early in development in rats (Nugent et al., 2015) and mice (Cisternas, Cortes, Bruggeman et al., 2020), and from late gestation into adulthood in humans (Numata et al., 2012; H. Xu et al., 2014). Future studies should test if ERα and *Tet2* similarly cooperate in the brain to regulate estrogen-responsive genes during the neonatal period of sexual differentiation. If so, ERα may bias the male brain to a hypomethylated state relative to its female counterpart.

All animals in this dissertation were examined prior to puberty in order to test the impact of neonatal manipulations of DNA methylation and demethylation in the absence of confounding effects of pubertal/adult hormones. While this was a strength of the experimental design, it also points to another limitation of this work. Our manipulations disrupted sexual differentiation, but we do not know if these effects are “permanent” or, at least, persist to adulthood. It would be

interesting to test if pubertal hormones are able to rescue any of the effects of neonatal inhibition of DNA methylation/demethylation on neural sex differences.

5.9 Implications for neurological disorders and diseases

Sex differences in the epigenome that occur during normal development may be relevant to understanding sex differences in neurological disorders and diseases, many of which exhibit sex differences in incidence, age of onset, or severity. For example, boys are more likely than girls to be diagnosed with developmental disorders, such as autism or attention deficit hyperactivity disorder (Boyle et al., 2011), whereas women are at an increased risk to suffer from anxiety, depression, post-traumatic stress disorders and Alzheimer's disease (Kessler et al., 1993; Tolin and Foa, 2006; Laws et al., 2018). Sex biases in disorders and diseases reflect sex differences in risk and/or protective factors, some of which are likely to be epigenetic.

A growing body of literature ties epigenetic changes associated with specific genes to developmental and adult onset neurological disorders (Nagarajan et al., 2006; Gonzales & LaSalle, 2010; Chouliaras et al., 2013; Tseng et al., 2014; Y. Xu et al., 2015). Other neurological disorders have been linked to gene mutations in histone modifying enzymes, DNMTs or methyl binding domain (MBD) proteins which act as the "readers" of DNA methylation by binding to mC (Jakovcevski & Akbarian, 2012). If one sex "relies" on a given epigenetic mechanism more than the other to control gene expression, then any disruption of that mechanism will affect the sexes differentially.

The work described in this dissertation establishes that sex differences are found in the mouse brain epigenome during a critical developmental period. Furthermore, we demonstrate sex-specific effects of developmental disruptions to DNA methylation or demethylation on the

establishment of neurochemical cell phenotype. This work may therefore provide insight into the basis of sex-specific vulnerability to developmental disorders linked to epigenetic processes.

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