Neonatal inhibition of DNA methylation disrupts testosterone-dependent masculinization of neurochemical phenotype

Carla D. Cisternas[1,2], Laura R. Cortes[1,2], Ilona Golynker[1], Alexandra Castillo-Ruiz[1] and Nancy G. Forger[1]

[1]Neuroscience Institute, Georgia State University, Atlanta, GA. USA 30302

[2]These authors contributed equally to this work.

Abbreviated Title: Masculinization of neurochemical phenotype

Keywords: epigenetic, sex difference, medial preoptic area, bed nucleus of the stria terminalis, anteroventral periventricular nucleus

Corresponding Author: Nancy G. Forger, Neuroscience Institute, Georgia State University, Atlanta, GA 30302, nforger@gsu.edu, 404-413-5888

Funding: Supported by a Brains & Behavior Seed Grant from Georgia State University (to NGF), and a National Science Foundation Graduate Research Fellowship (to LRC). CDC was supported by the Georgia State University Next Generation New Scholars Program.

Disclosure Statement: The authors have nothing to disclose.

ABSTRACT

Many neural sex differences are differences in the number of neurons of a particular phenotype. For example, male rodents have more calbindin-expressing neurons in the medial preoptic area (mPOA) and bed nucleus of the stria terminalis (BNST), and females have more neurons expressing estrogen receptor alpha (ERα) and kisspeptin in the ventromedial nucleus of the hypothalamus (VMH) and the anteroventral periventricular nucleus (AVPV), respectively. These sex differences depend on neonatal exposure to testosterone, but the underlying molecular mechanisms are unknown. DNA methylation is important for cell phenotype differentiation throughout the developing organism. We hypothesized that testosterone causes sex differences in neurochemical phenotype via changes in DNA methylation, and tested this by inhibiting DNA methylation neonatally in male and female mice, and in females given a masculinizing dose of testosterone. Neonatal testosterone treatment masculinized calbindin, ERα and kisspeptin cell number of females at weaning. Inhibiting DNA methylation with zebularine increased calbindin cell number only in control females, thus eliminating sex differences in calbindin in the mPOA and BNST. Zebularine also reduced the sex difference in ERα cell number in the VMH, in this case by increasing ERα neuron number in males and testosterone-treated females. In contrast, the neonatal inhibition of DNA methylation had no effect on kisspeptin cell number. We conclude that testosterone normally increases the number of calbindin cells and reduces ERα cells in males through orchestrated changes in DNA methylation, contributing to, or causing, the sex differences in both cell types.

INTRODUCTION

Many sex differences in the mammalian brain are established by a transient, perinatal exposure to gonadal testosterone in males (1–3). In some cases, testosterone regulates neuronal cell death to cause sex differences in neuron number (4,5); 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, (6,7)] and vasopressin in the bed nucleus of the stria terminalis [BNST, (8,9)], whereas females have more neurons expressing tyrosine hydroxylase and kisspeptin in the anteroventral periventricular nucleus and neighboring rostral periventricular nucleus [AVPV/PeN, (10–12)]. These sex differences all persist in mice lacking the pro-death gene *Bax* (13–16), despite the near complete elimination of developmental neuronal cell death in *Bax* knockout mice (17,18).

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 (19–21). DNA cytosine methylation is controlled by a family of DNA methyltransferases (DNMTs) that place methyl marks, and ten-eleven translocases (TET enzymes) that remove those marks (22–24). DNA methylation is normally associated with gene repression, although there are exceptions (25). The expression of DNMT enzymes peaks during the first postnatal week in the mouse brain (26,27), which coincides with the critical period for testosterone-dependent sexual differentiation. Moreover, there are sex differences in DNMT and TET activity and/or expression in the neonatal brain (21,26). We therefore hypothesized that sex differences

67 in neurochemical phenotype (i.e., the number of cells expressing specific markers) may depend
68 on differential DNA methylation in males and females.

69 In a first test of this idea (28), we previously administered a DNMT inhibitor to newborn
70 male and female mice, and examined effects on the male-biased sex difference in calbindin cell
71 number in the mPOA, and the female-biased sex difference in the number of estrogen receptor
72 (ER) α cells in the ventrolateral portion of the ventromedial hypothalamus [VMHvl, (29–31)].
73 The neonatal inhibition of DNA methylation increased the number of cells expressing both cell
74 types at weaning (28), consistent with the canonical association of DNA methylation with the
75 suppression of gene transcription, and also reduced or eliminated the sex differences in calbindin
76 and ERα cell number (28).

77 Calbindin cell number in the mPOA is masculinized in female rats and mice treated with
78 testosterone or estradiol at birth, and the sex difference is present prior to puberty (15,32,33).
79 The sex difference in ERα in the VMHvl is also evident prior to puberty in rats and mice
80 (28,31,34), although its dependence on neonatal testosterone has not yet been demonstrated.
81 Here, we hypothesized that testosterone causes these sex differences in cell phenotype (a
82 decrease in ERα and an increase in calbindin) by orchestrating changes in DNA methylation
83 around the time of birth. If so, then effects of endogenous or exogenous testosterone may be
84 prevented by inhibiting DNA methylation.

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

greater in males, (15)] and kisspeptin cell number in the AVPV/PeN [greater in females; (10,16)]. We find that neonatal inhibition of cytosine methylation eliminates or reduces sex differences in calbindin and ERα. Interestingly, it does so by increasing cell counts 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).

MATERIALS AND METHODS

Animals

Wildtype C57BL6/J 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 (LabDiet 5015, St. Louis, MO, USA) 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.

Zebularine injections

DNA methylation was inhibited using zebularine (Calbiochem, San Diego, CA), a cytidine analog and global DNMT inhibitor that has been used in many rodent studies due to its low toxicity (35,36). 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. This dose was chosen based on our own previous work and that of others (21,28). 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).

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 as in (37)] or the oil vehicle on P0 and P1; all males received the vehicle only. Animals in each group were derived from at least six different litters, and were sacrificed at weaning on P25, as previously (28), 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.

Immunohistochemistry for calbindin, ERα, and kisspeptin

One series of sections was stained for calbindin [mouse anti-calbindin, 1:20,000 anti-calbindin-D28k; Sigma; (38)], one for ERα [rabbit anti-ERα, 1:20,000; EMD Millipore, Billerica, MA; (39)], and one for kisspeptin [rabbit anti-kisspeptin, 1:2,000; EMD Millipore;(40)]. Protocols are described in detail elsewhere (28). 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 (41)], or biotinylated goat anti-rabbit [1:250 for ERα and 1:500 for kisspeptin, Vector Laboratories (42)]. Staining was visualized using an avidin-biotin complex followed by incubation in diaminobenzidine-nickel (Vector Laboratories).

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 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 [Figures 31-34 in the Paxinos & Franklin mouse brain atlas (43)]. Labeled cells within the contours were counted in the left and right hemispheres of at least two brain sections and the two highest counts were summed, as previously (15). Calbindin-positive cells in the encapsulated portion of the BNST [Figure 31 in (43)] were quantified as previously (44) 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 based on the characteristic shape and location of the nucleus [Figures 42-47 in (43)], 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 identified using the anterior commissure and third ventricle as landmarks [Figures 29-33 in (43)], and all labeled cells in all sections were counted. Animals for which the sections of interest were

damaged, folded, or missing were omitted from the analysis (final *N* in each group is indicated at the base of each bar in the figures).

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-P2). 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.

Statistical analyses

Data were checked for normality and homogeneity of variance using IBM SPSS Statistics. DNMT activity after zebularine injections was analyzed using two-tailed independent t-tests. *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, and $P < 0.05$ was considered significant.

RESULTS

Zebularine transiently decreases global DNMT activity

Zebularine reduces DNA methylation within one hour in hippocampal slice cultures (45), and icv injections to adult rats reduce DNA methylation in the brain within four hours (46). 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 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 1). Thus, zebularine transiently decreased global DNMT activity.

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). 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

203 < 0.01) on calbindin cell number in the two-way ANOVA (Figure 2B). Calbindin cell number
204 was significantly higher in control males and testosterone-treated females than in control females
205 ($P < 0.0001$ for both comparisons). Neonatal zebularine treatment increased calbindin cell
206 number at weaning only in control females ($P < 0.02$) and was as effective as testosterone in this
207 regard (female + testosterone vs female + zebularine, $P = 0.66$). As a result, group differences
208 were abolished in zebularine-treated mice.

209 The same general pattern was seen for calbindin cells in the BNST. We confirmed that
210 the sex difference in calbindin cell number previously seen in the BNST of *adults* (15) is present
211 prior to puberty (control male versus control female, $t_{21} = 2.23$; $P < 0.04$; Figure 3). There was a
212 trend for a higher number of calbindin-positive cells in the female + testosterone group compared
213 to control females, but this did not reach significance ($P < 0.1$). By two-way ANOVA, we found
214 a significant effect of zebularine treatment on calbindin cell number ($F_{1, 61} = 4.02$, $P < 0.05$;
215 Figure 3B): zebularine increased the number of calbindin-positive neurons overall, and within
216 groups this was significant only for females ($P < 0.05$).

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

219

220 ***Neonatal inhibition of DNA methylation partially prevents the masculinizing effect of***
221 ***testosterone on ERα cell number***

222 In contrast to the male-biased sex differences in calbindin cell number, females have
223 more ERα neurons in the VMHvl than do males. We confirmed this sex difference and found
224 that neonatal testosterone decreased ERα cell number at weaning in females (control female vs
225 testosterone-treated female, $t_{13} = 9.89$; $P < 0.0001$) to a level indistinguishable from that in males

(Figure 4). In the ANOVA, we found significant main effects of group ($F_{2, 53} = 80.1$, $P < 0.0001$) and zebularine treatment ($F_{1, 53} = 4.75$, $P = 0.034$), as well as a group-by-treatment interaction ($F_{2, 53} = 5.03$, $P = 0.01$; Figure 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 ($P < 0.03$ 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.

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 5), 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 ($F_{1, 47} = 3.30$, $P = 0.076$).

DISCUSSION

Neonatal testosterone (or its estrogenic metabolites) can alter DNA methylation patterns in the brain (21,47,48). 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. Zebularine did not fully increase ERα cell number in males and testosterone-treated females to female-like levels, however. This may be related to the fact that the inhibition of DNMT activity we achieved was partial (a 54% reduction at 6 h), and a more profound inhibition may be required for female-like development of the ERα phenotype. Alternatively, mechanisms other than cytosine methylation may be involved; this might, for example, include histone modifications, or non-cytosine DNA methylation. Recently, methylation of other bases (especially, adenine) has been demonstrated in neurons (27) and zebularine, a cytidine analog, would not be expected to inhibit adenine methylation.

An increase in cell number after neonatal zebularine treatment could, in principle, be due to a change in cell phenotype (i.e., cells now express the marker of interest) or a decrease in developmental cell death (i.e., more cells survive). The evidence in favor of a change in cell phenotype is strong for the calbindin cell groups examined here. First, sex differences in calbindin cell number in the mPOA and BNST persist even when developmental cell death is prevented (15). In addition, we previously found no change in developmental cell death and no change in total cell number in the mPOA at weaning after neonatal zebularine treatment (28). Thus, early-life inhibition of DNA methylation changes the number of cells that express calbindin, without changing total cell number. We also found no effect of neonatal zebularine treatment on cell death in the VMHvl (28). However, total cell number in the VMHvl was not examined, and the ERα sex difference has not been examined in cell death mutant mice. Thus, the conclusion that zebularine changes cell phenotype independent of a change in cell number for ERα is more tentative, and awaits confirmation.

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 (26,27,48,49). Previous studies have shown that pharmacological perturbations to epigenetic mechanisms do not globally affect the genome, but may particularly target genes undergoing active regulation (50). 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 (21). 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 (21,26), as well as lower expression of the TET enzymes that are responsible for de-methylation (26). 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 (30,31), and TET enzyme expression is higher in males than in females in the neonatal VMH (26), 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 [(51–53)], 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 (10). In rats, the sex difference in kisspeptin cell number in the AVPV/PeN results from early life exposure to testosterone and its estrogenic metabolites (11,54), 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. (55) 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 (55). Taken together with the current study, there 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, and that DNA methylation underlies both feminization [as shown by calbindin cell number in the present study and (21)], and masculinization [ERα cell number in the present study and (28)] of neuronal cell phenotype. 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 various projections and functions (56–60). Males and females start out with an equally high number of ERα neurons in the VMHvl at birth (28), and we are currently examining whether the sex difference that emerges by weaning is 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.

FIGURE LEGENDS

Figure 1: 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. The number of animals per group is indicated at the base of each bar.

Figure 2: 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 (gray horizontal lines with asterisks). Neonatal treatment with zebularine increased CALB+ cell number only in females (black horizontal line) and eliminated group differences. The number of animals per group is indicated at the base of each bar. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$. Data are mean ± SEM.

Figure 3: 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. The number of animals per group is indicated at the base of each bar. * $P < 0.05$. Data are mean ± SEM.

Figure 4: 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). 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. Gray horizontal lines with asterisks indicate significant effects of sex and black horizontal lines indicate significant effects of zebularine. The number of animals per group is indicated at the base of each bar. * $P < 0.05$; **** $P < 0.0001$. Data are mean ± SEM.

Figure 5: 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 of kisspeptin+ cells in the AVPV/PeN at weaning in males, females, and testosterone-treated females that received icv vehicle or zebularine at birth. 3V: third ventricle. B) Quantification reveals that females had 40-

400 fold more kisspeptin-positive cells than did males or testosterone-treated females at weaning.
401 Neonatal zebularine treatment did not significantly affect kisspeptin cell number. Gray
402 horizontal bars indicate significant effects of sex . The number of animals per group is indicated
403 at the base of each bar. **** $P < 0.0001$. Data are mean ± SEM.

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