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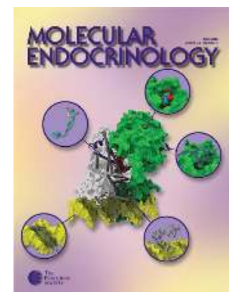

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Epigenetic Control of Sexual Differentiation of the Bed Nucleus of the Stria Terminalis

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The principal nucleus of the bed nucleus of the stria terminalis (BNSTp) is larger in volume and contains more cells in male than female mice. These sex differences depend on testosterone and arise from a higher rate of cell death during early postnatal life in females. There is a delay of several days between the testosterone surge at birth and sexually dimorphic cell death in the BNSTp, suggesting that epigenetic mechanisms may be involved. We tested the hypothesis that chromatin remodeling plays a role in sexual differentiation of the BNSTp by manipulating the balance between histone acetylation and deacetylation using a histone deacetylase inhibitor. In the first experiment, a single injection of valproic acid (VPA) on the day of birth increased acetylation of histone H3 in the brain 24 h later. Next, males, females, and females treated neonatally with testosterone were administered VPA or saline on postnatal d 1 and 2 and killed at 21 d of age. VPA treatment did not influence volume or cell number of the BNSTp in control females but significantly reduced both parameters in males and testosterone-treated females. As a result, the sex differences were eliminated. VPA did not affect volume or cell number in the suprachiasmatic nucleus or the anterodorsal nucleus of the thalamus, which also did not differ between males and females. These findings suggest that a disruption in histone deacetylation may lead to long-term alterations in gene expression that block the masculinizing actions of testosterone in the BNSTp. **(*Endocrinology* 150: 4241–4247, 2009)**

The bed nucleus of the stria terminalis is a sexually dimorphic limbic forebrain structure involved in the control of male sex behavior, gonadotropin release, and the modulation of stress and anxiety (1–5). The principal nucleus of the bed nucleus of the stria terminalis (BNSTp) is larger and contains more cells in males than females of several species, including humans (6–10). In rats and mice, sexually dimorphic cell death during the first week of life can account for the sex difference in cell number in the BNSTp seen in adulthood (7, 11, 12), and this sex difference can be eliminated by neonatal castration of males or treating females with testosterone propionate (TP) at birth (6, 13).

A single neonatal injection of TP leads to a reduction of cell death in females about 5 d later (12). This suggests a cellular memory for the early hormone exposure consistent with changes in the epigenome. A number of epigenetic processes have been identified, including DNA methylation and covalent modifications of histones (14). Steroid hormones can induce such chromatin modifications to bring about long-lasting changes in gene expression in cancer cell lines, peripheral tissues, and the brain (15–18).

Of the many possible histone modifications, acetylation is currently the best understood in terms of effects on gene transcription. Addition of acetyl groups to lysine residues on histone tails is catalyzed by histone acetyltransferases (HATs) and is most commonly associated with transcriptional activation. Conversely, histone deacetylation is generally associated with repression of transcrip-

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Abbreviations: AcH3, Acetylated histone H3; AD, anterodorsal nucleus of the thalamus; BNSTp, principal nucleus of the bed nucleus of the stria terminalis; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; HAT, histone acetyltransferase; HDAC, histone deacetylase; SCN, suprachiasmatic nucleus of the hypothalamus; TBS, Tris-buffered saline; TP, testosterone propionate; VPA, valproic acid.

tion (19–21), although there are some important exceptions (22–24). Several of the best-known steroid hormone receptor coactivators have HAT activity or recruit HATs to the transcription complex (25–27), whereas corepressors often have histone deacetylase (HDAC) activity. Thus, changes in histone acetylation are likely to be important for many effects of steroid hormones.

We hypothesized that the effects of testosterone on sexual differentiation of the brain requires orchestrated changes in histone acetylation. To test this, we administered an HDAC inhibitor, valproic acid (VPA), to neonatal mice during the critical period for sexual differentiation and examined the volume and cell number of the BNSTp and two control regions at 3 wk of age. Our results suggest that neonatal disruption of histone deacetylation blocks masculinization of the BNSTp.

Materials and Methods

Animals

Wild-type C57BL/6 mice from our breeding colony were housed under 14-h light, 10-h dark conditions at 22 C. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Massachusetts (Amherst, MA).

Tissue preparation for immunoblotting

Mice were injected sc with 50 mg/kg VPA on the day of birth and killed by rapid decapitation at 6, 12, 24, 48, 72, or 96 h after injection. Five males and five females were collected at each time point. Brains were removed and rapidly frozen with 2-methylbutane kept at −80 C. One hemisphere of each brain was homogenized over ice in 30 volumes of radioimmunoprecipitation assay buffer [0.05 M Tris, 1% Igepal CA-630, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Sigma, St. Louis, MO)]. Protein was extracted at 4 C for 30 min followed by centrifugation at the same temperature for 20 min at 15,000 × *g*. The resulting supernatant was aliquoted and stored at −80 C before SDS-PAGE analysis. Total protein concentration was determined with the BCA protein assay (Pierce, Rockford, IL) using BSA as the standard.

SDS-PAGE

Equal amounts of protein (15 μg/lane) from each sample were combined with loading buffer and boiled for 5 min. Samples were run on a 12% precast Tris-HCl polyacrylamide gel (Bio-Rad, Hercules, CA), followed by protein electrotransfer at 100 V for 25 min onto a polyvinylidine difluoride membrane (Bio-Rad). Membranes were then rinsed in 0.05 M Tris-buffered saline (TBS), followed by blocking in 5% nonfat dry-milk in TBS for 1 h. Acetylation of histone H3 is often used as a measure of HDAC inhibition (28). Membranes were therefore probed with an antibody directed against acetylated histone H3 (AcH3; Upstate, Temecula, CA) overnight at 4 C. This antibody recognizes acetylated Lys-9 and Lys-14 on histone 3 and has previously been used to detect AcH3 in the mouse brain (29). Membranes were rinsed with TBS containing 0.2% Tween 20 and incubated with a secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. After washing in TBS containing 0.2% Tween 20, membranes were immersed in enhanced chemiluminescent reagent (ECL-Plus; Amersham Biosciences, GE Healthcare, UK), and the resulting chemiluminescent reaction was exposed to film (Biomax MR; Kodak, Rochester, NY). Membranes were subsequently stripped and reprobed with an antibody against β-actin (Sigma) to verify equal loading of proteins.

OD of protein bands was determined using Image J densitometry software (National Institutes of Health, Bethesda, MD). The OD of the AcH3 band was divided by the OD of the β-actin band for each sample. Equal numbers of saline and VPA-treated animals were run on each gel, and data were expressed as percent of saline control.

Testosterone and VPA treatments

We first conducted a pilot study to determine an appropriate dose of VPA for neonatal mice. Doses used routinely in adult mice (100–500 mg/kg) often caused pup mortality. In contrast, 50 mg/kg VPA was well tolerated by pups and led to reliable changes in histone acetylation in the brain (see below).

Females received TP (100 μg in 25 μl peanut oil) or an equal volume of oil on the day of birth; males were treated with oil. Half of the animals in each of the three hormonal conditions also were treated with VPA (50 mg/kg in 0.9% saline) or saline on postnatal d 1 and 2. Two daily doses of VPA were administered to overlap with the expected period of elevated testosterone after TP injection [>24 h (30)]. Animals were weighed and killed by CO_2 inhalation at 3 wk of age, after the period of sexually dimorphic cell death (11). Brains were removed, postfixed in 5% acrolein in 0.1 M phosphate buffer for 4 h, and immersed in 30% sucrose until sectioning. Brains were cut (40 μm) in the coronal plane on a freezing microtome and alternate sections were mounted, dehydrated, and stained with thionin.

BNSTp volume and cell counts

All measurements were made on slides coded to conceal the sex and treatment of the animals. StereoInvestigator software (MicroBrightfield, Williston, VT) was used to measure nuclear volume and perform stereological cell counts as previously described (7). The outline of the BNSTp was traced bilaterally in every section in which it appeared, and volume was calculated by multiplying the summed area by the sampling ratio and section thickness. The optical dissector method was used to perform stereological counts. A counting frame of 16 × 16 μm was systematically moved throughout the traced region of the BNSTp, with counts performed every 75 μm (*i.e.* a sampling grid of 75 × 75 μm). Upper and lower guard zones of 1 μm were used to avoid edge artifacts. Cells with a visible nucleus that fell within the counting frame and had the morphological characteristics of neurons were counted. Volume measurements and cell counts were also carried out for two control regions, the suprachiasmatic nucleus of the hypothalamus (SCN) and the anterodorsal nucleus of the thalamus (AD). These regions were chosen because they have clear boundaries, occur in the same sections as the BNSTp, and were not expected to be sexually dimorphic.

Statistical analysis

There was no effect of sex on histone acetylation in the brain after VPA treatment in a three-way ANOVA (sex by time by

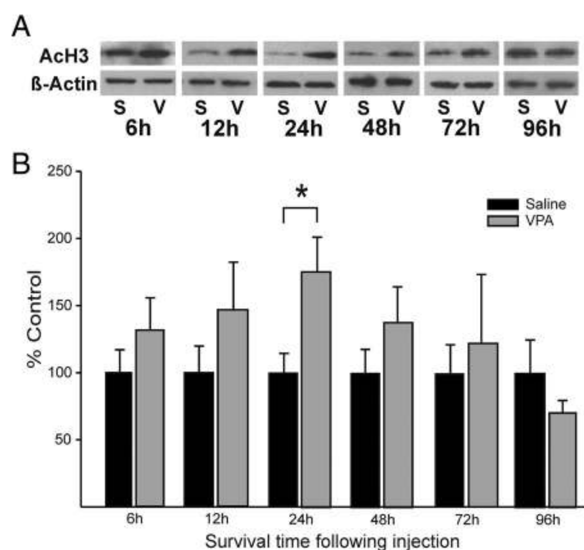

FIG. 1. VPA increases AcH3 in the mouse brain. A, Western blots showing levels of AcH3 and β-actin 6, 12, 24, 48, 72, or 96 h after saline (S) or VPA (V) treatment. Data from males and females were combined for this analysis. B, Mean ± SEM level of AcH3 corrected for β-actin and expressed as percent of saline control for each time-point (n = 8–10/group). VPA significantly increased AcH3 24 h after injection. *, $P < 0.05$ and across all time points combined (main effect of treatment, $P < 0.05$).

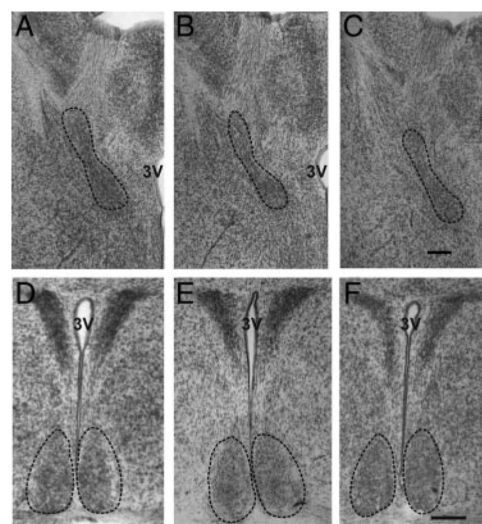

FIG. 2. Representative photomicrographs through the BNSTp (*dotted line*, A–C) and SCN (*dotted line*, D–F) of a control male (A and D), control female (B and E), and VPA-treated male (C and F). Volume of the BNSTp was larger in males than females. Masculinization of BNSTp volume was blocked by VPA treatment. There was no effect of sex or treatment on the volume of the SCN. *Scale bar* in C, 100 μm for A–C; in F, 200 μm for D–F. 3V, Third ventricle.

treatment). Therefore, data from males and females were combined in subsequent analyses. Effects of VPA on histone acetylation were tested with a two-way ANOVA (treatment by time), followed by planned comparisons of individual time points. Volume and cell number in the BNSTp, SCN, and AD were also analyzed using two-way ANOVAs (group by drug treatment). Planned comparisons used Fisher's least significant differences.

Results

VPA increases AcH3 in the brain

Levels of AcH3 in the brain were measured at six time points after a single injection of 50 mg/kg VPA on the day of birth (Fig. 1). ANOVA revealed a significant overall effect ($P < 0.05$), with higher levels of AcH3 in VPA-treated animals. Although AcH3 levels appeared elevated at 6, 12, 24, and 48 h after injection, planned comparisons at individual time points indicated that the difference reached significance only at 24 h ($P < 0.05$; Fig. 1). Thus, a single injection of VPA on the day of birth causes a transient increase in AcH3.

VPA blocks masculinization of BNSTp volume and cell number

As predicted, volume and cell number in the BNSTp were significantly greater in oil-treated males than oil-treated females on postnatal d 21 (volume: $P < 0.0001$; cell number: $P < 0.0001$, Figs. 2 and 3). In females treated with TP at birth, BNSTp volume and cell number were increased relative to oil-treated females (volume: $P < 0.0001$; cell number: $P < 0.0001$, Fig. 3) and did not differ from males on either measure ($P > 0.6$).

Treatment with VPA reduced overall volume and cell number in the BNSTp of males (volume: $P < 0.05$; cell number: $P < 0.0001$) and TP-treated females (volume: $P = 0.01$; cell number: $P < 0.0001$; Figs. 2 and 3) but had no effect on these measures in oil-treated females ($P > 0.3$). Males and TP females treated with VPA did not differ from control females on any measure. Thus, masculinization of the BNSTp was blocked by VPA treatment. These findings were reflected in significant group-by-treatment interactions in the two-way ANOVAs (volume: $P < 0.005$; cell number: $P < 0.0001$).

There were no sex differences and no effect of VPA treatment in the SCN (Figs. 2 and 3) or AD (data not shown). VPA also had no effect on body weight; treated animals, if anything, were slightly (nonsignificantly) heavier than controls (saline: 9.57 ± 0.36 g; VPA: 9.93 ± 0.19 g; $P > 0.25$).

Discussion

Several recent studies provided evidence for sex differences in epigenetic alterations in the brain. In rats, levels of the DNA methyl binding protein, MeCP2, are transiently elevated in the amygdala and ventromedial hypothalamus of females at birth (31), and decreasing MeCP2 expression in the amygdala prevents sex differences in juvenile social play (32). In addition, levels of acetylated and trimethylated histone H3 are sexually dimorphic (greater in males)

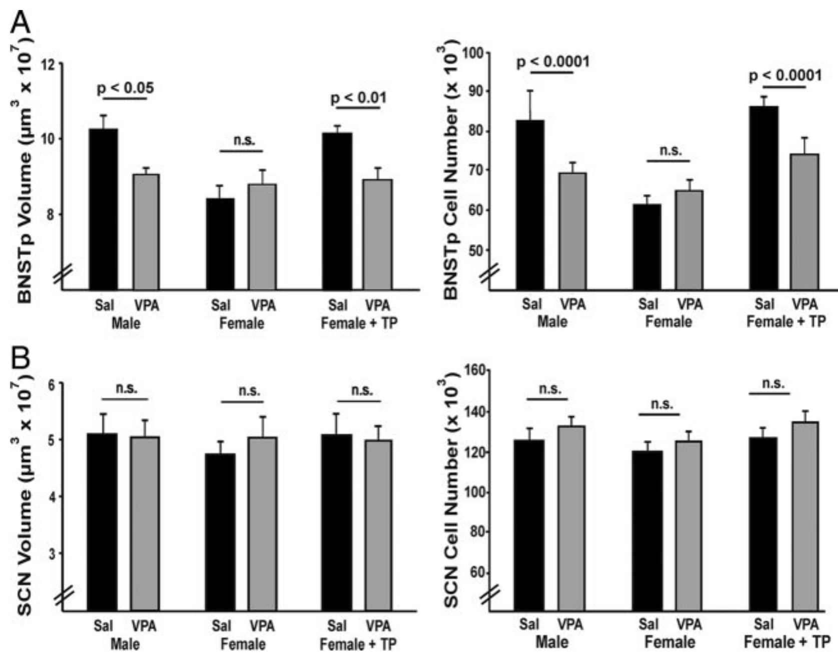

FIG. 3. Effect of VPA treatment on volume and cell number in the BNSTp and SCN of males, females, and TP-treated females treated with saline (Sal) or VPA on postnatal d 1 and 2 (n = 9–11/group). A, Volume and cell number of the BNSTp were greater in males and TP-treated females than oil-treated females. VPA blocked masculinization of the BNSTp in males and TP-treated females but did not affect BNSTp volume or cell number in control females. B, VPA had no effect on volume or cell number in the SCN in any group. n.s., Not significant.

in the cortex and hippocampus of perinatal mice (33). The functional consequences of these sex differences are unknown, and to date, no study has manipulated epigenetic processes and subsequently examined the effect on sexual differentiation of brain morphology. Here we report that treatment with a histone deacetylase inhibitor during the critical period for sexual differentiation prevents masculinization of the BNSTp in mice. Males and androgenized females treated neonatally with VPA had female-like BNSTp volume and cell number. Importantly, effects of VPA depended on hormonal status because VPA had no effect on the BNSTp of females in the absence of testosterone. Taken together, our data are consistent with the hypothesis that testosterone acts through epigenetic processes, in particular the regulation of histone acetylation, to direct sexual differentiation of the brain.

A growing number of histone deacetylase inhibitors are currently available. VPA was chosen in this study primarily for its long half-life *in vivo*: approximately 12 h in adults and longer in newborns (34). By contrast the commonly used HDAC inhibitor trichostatin A has a half-life less than 10 min in mice (35). We find that a single injection of VPA at birth increases levels of acetylated histone H3 in the brain 24 h later, suggesting that systemic VPA treatment of pups can disrupt brain histone acetylation patterns during the critical period for sexual differentiation.

However, several alternative explanations for our results must be considered. Like all HDAC inhibitors, VPA has activities independent of HDAC inhibition (36). For example, long-term administration of VPA increases γ-aminobutyric acid (GABA) levels in the brain (37), and GABA has been implicated in sexual differentiation (38). Male rats have higher levels of the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) than do females in some regions of the hypothalamus and hippocampus neonatally (39), and a blockade of GAD reduces male sexual behavior in androgenized females (39, 40). As far as we know, sex differences in GAD or GABA have not been reported in the bed nucleus of the stria terminalis. Moreover, if our findings were due to effects on GABA, one would predict masculinization in response to VPA treatment, whereas the opposite was found.

VPA also may have teratogenic effects on developing rodents (41, 42). The studies reporting such effects use doses of VPA approximately 10-fold higher than that used here and administer VPA much earlier in development. In addition, although VPA can be cytotoxic to cancer cell lines (43–45), it is often protective to neurons (44, 46–49). Importantly, BNSTp volume and cell number were unchanged in females treated with VPA in the current study, and there was no effect of treatment on volume or cell number in two control regions. These observations argue against a nonspecific or toxic effect of VPA and instead suggest a specific interaction between VPA treatment and hormone status.

It is not yet known how VPA blocks masculinization of the BNSTp. A generalized disruption of histone deacetylation could potentially exert widespread effects on the epigenome, although in normal cell populations, treatment with an HDAC inhibitor alters the expression of a relatively small number of genes. For example, less than 2% of more than 30,000 genes surveyed in the hippocampus were changed by HDAC inhibition *in vivo* (50). Interestingly, several investigators have noted that genes regulated by HDAC inhibitors fall predominantly into two categories: those related to cell cycle and apoptosis (51, 52). This suggests that cells undergoing active regulation may be particularly sensitive to HDAC inhibition.

It is difficult to predict the direction of changes in gene expression after VPA treatment. Although HDAC inhibition might be expected to increase the expression of genes, in fact, treatment with an HDAC inhibitor leads to nearly equal numbers of genes with increased *vs.* decreased expression (53–55). This is perhaps not surprising because the protein products of some genes will suppress the expression of other genes. In addition, for some genes an increase in histone acetylation directly inhibits expression (22, 23, 56). The sex difference in cell number in the BNSTp is determined by hormone-dependent sexually dimorphic cell death during development and requires *bax*, a prodeath member of the *bcl-2* family of apoptosis-related genes (7, 11). In the neonatal rat preoptic area, testosterone decreases Bax but increases Bcl-2 protein expression (57). Therefore, a disruption of the hormonal regulation of *bax* or other *bcl-2* family genes is a potential mechanism of VPA action in the current study; future studies will identify direct and indirect gene targets of neonatal VPA administration.

Understanding the epigenetic regulation of cell number in the BNSTp in response to hormones may have important clinical implications. The BNSTp plays a vital role in modulating emotional and stress responses, with lesions increasing the hypothalamic-pituitary-adrenal response to acute stress (58, 59). This indicates that the BNSTp normally reduces stress responsivity and suggests that the larger BNSTp in males may contribute to the known sex difference in stress responsiveness (60). Because VPA eliminated sex differences in the BNSTp in the current study, it would be interesting to test the effects of neonatal VPA treatment on sex differences in the hypothalamic-pituitary-adrenal axis and anxiety-related behaviors. In humans, women are more susceptible to mood disorders than men (60, 61), and there are sex differences in the prevalence of most psychiatric illnesses. Interestingly, gender-specific epigenetic modifications have recently been reported in the brains of patients with schizophrenia and bipolar disorder (62). This suggests that sex differences in the epigenome may underlie sex biases in the incidence of neurological disorders.

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