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Inhibition of DNA Methylation in the Developing Rat Brain Disrupts Sexually Dimorphic Neurobehavioral Phenotypes in Adulthood

Yong Li¹, Qingyi Ma¹, Chiranjib Dasgupta¹, Shina Halavi², Richard E. Hartman², Daliao Xiao¹, and Lubo Zhang¹

¹Center for Perinatal Biology, Division of Pharmacology, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA 92350, USA

²Department of Psychology, Loma Linda University, Loma Linda, CA 92354, USA

Abstract

Abstract Accumulating evidence indicates a critical implication of DNA methylation in the brain development. We aim to determine whether the disruption of DNA methylation patterns in the developing brain adversely affects neurobehavioral phenotypes later in life in a sex-dependent manner. 5-Aza-2'-deoxycytidine (5-Aza), a DNA methylation inhibitor, was administered in newborn rats from postnatal day 1 to 3. Neurobehavioral outcomes were analyzed at 3 months of age. 5-Aza treatment significantly inhibited DNA methyltransferase activity and decreased global DNA methylation levels in neonatal rat brains, resulting in asymmetric growth restriction with the increased brain to body weight ratio in both male and female rats at 14 days and 3 months of age. Compared with the saline control, 5-Aza treatment significantly improved performance of male rats on the rotarod test, and 5-Aza-treated female rats demonstrated less anxiety, less depression-like behaviors, and enhanced spatial learning performance. Of importance, neonatal 5-Aza treatment eliminated the sexually dimorphic differences in several neurobehavioral tests in adult rats. In addition, 5-Aza treatment decreased promoter methylation of brain-derived neurotrophic factor (BDNF) gene and significantly increased BDNF mRNA and protein abundance in the prefrontal cortex and hippocampus of female rats in a sex-dependent manner. Thus, brain DNA methylation appears to be essential for sexual differentiations of the brain and neurobehavioral functions. Inhibition of DNA methylation in the developing brain of early life induces aberrant neurobehavioral profiles and disrupts sexually dimorphic neurobehavioral phenotypes in adulthood, of which altered BDNF signaling pathway may be an important mediator.

✉ Lubo Zhang, lzhang@llu.edu.

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Compliance with Ethical Standards All experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Loma Linda University.

Conflict of Interest The authors declare that they have no conflict of interest.

Keywords

DNA methylation; 5-Aza; Neonatal brain; Sexual differentiation; Neurobehavioral phenotypes; BDNF

Introduction

Epigenetic mechanisms are essential regulators of gene expression that do not alter the DNA sequence, allowing the integration of intrinsic and extrinsic signals into sustained changes in gene expression and facilitating the adaptation of an organism to its changing environment through alterations in gene activity [1–4]. It has been recognized that epigenetic regulation is a critical mechanism in a wide range of behavioral development including processes involved in learning and memory [5, 6]. DNA methylation is the most characterized epigenetic mechanism and is an inherently stable mark incapable of rapid change [7, 8]. Properly established and maintained patterns of DNA methylation are essential for normal brain development, cell lineage commitment, and neural functions [9–11]. Pathological DNA methylation profiles, both hypomethylation or hypermethylation, may result in altered gene expression patterns and thus contribute to multiple brain pathologies in various neurological and psychiatric conditions [12–15].

Large amount of evidence has suggested that early life adverse experiences during a critical developmental period can contribute to the programming of behavioral phenotypes later in life by altering behavioral trajectories and causing aberrant behavioral outcomes. These may include certain neuropsychiatric disorders, of which brain DNA methylation profiles play an important role [16–18]. There are many examples of neuroanatomical and behavioral differences between the genders, which also differ in their susceptibility to a broad range of brain diseases [19–21]. Sexual differentiation of the brain is a crucial developmental process that occurs during a critical window of the perinatal development as a result of gonadal hormone-induced activation and organizational effects on neuronal substrates, enabling the lifelong expression of sexually dimorphic behaviors [21, 22]. Increasing studies suggest that epigenetic mechanisms including DNA methylation contribute to the establishment and maintenance of some of these processes [19, 21–23].

The present study characterized the effects of inhibition of neonatal brain DNA methylation on a series of neurobehavioral phenotypes in male and female adult rats. Herein, we present novel evidence that DNA methylation profiles in the developing brain are related to sexual differentiation of the brain and behavior later in life. Inhibition of DNA methylation in the early life not only induces abnormal neurobehavioral outcomes but also disrupts sexually dimorphic differentiation of neurobehavioral phenotypes in adulthood, of which brain-derived neurotrophic factor (BDNF)-TrkB signaling may act as a critical mediator.

Methods

Experimental Animals

Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Portage, MI). Animals were allowed to give birth, and pups of both sexes were randomly divided into

the saline control and 5-aza-2'-deoxycytidine (5-Aza)-treated groups. Saline or 5-Aza (1 mg/kg) was administered to newborn rats via intraperitoneal (i.p.) injection consecutively for 3 days from postnatal day 1 to 3 (P1–3). Total of 11 litters were included. No pup died in the 5-Aza treatment. Brains were collected from day 14 (P14) pups and adult rats for molecular studies of global DNA methylation, DNA methyltransferase (DNMT) activity, the expressions of DNMT1, 3a and 3b, promoter methylation of brain-derived neurotrophic factor (BDNF) gene, and BDNF messenger RNA (mRNA) and protein abundance. Neurobehavioral tests were conducted in adult animals in 3 months of age. Animals were randomly assigned to four groups including saline-treated males, 5-Aza-treated males, saline-treated females, and 5-Aza-treated females, each group including 10 rats from different litters. Animals were kept in a 12-h light/dark cycle and provided ad libitum access to normal rat chow and filtered water. All procedures and protocols were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

5-Methylcytosine DNA ELISA

Genomic DNA was isolated from whole rat brains on P14, and global DNA methylation was determined by measuring 5-methylcytosine (5-mC) using a 5-mC DNA ELISA Kit (Zymo Research), as described previously [24]. Briefly, 100 ng of genomic DNA and standard controls provided by the kit were denatured and used to coat the plate wells with 5-mC coating buffer. After incubation at 37 °C for 1 h, the wells were washed with 5-mC ELISA buffer, and then, an antibody mix consisting of anti-5-mC and a secondary antibody was added to each well. The plate was covered with foil and incubated at 37 °C for 1 h. After the antibody mix was washed out from the wells with the 5-mC ELISA buffer, a HRP developer was added to each well and incubated at room temperature for 1 h. The absorbance at 405 nm was measured using an ELISA plate reader. The percent 5-mC was calculated using the second-order regression equation of the standard curve that was constructed with negative control and positive controls in the same experiment.

DNMT Activity Assay

DNMT activity assay was performed using an ELISA EpiQuik DNMT Activity Assay Kit (Epigentek), as described previously [25]. Briefly, nuclear extracts (NE) isolated from brains were incubated with S-adenosylmethionine (AdoMet) and a universal proprietary DNMT substrate in the DNMT assay buffer at 37 °C for 2 h. The blank contained only AdoMet and the substrate without NE, while the positive control contained AdoMet/substrate with the purified DNMT enzyme preparation containing both maintenance and de novo DNMTs, supplied in the kit. After the incubation, the capture antibody and detection antibody were added in sequence, followed by incubation with developer solution for 10 min at room temperature. Signal was measured by a dual-wavelength microplate reader at 450/655 nm. The DNMT activity [optical density (OD)/h/mg] was calculated by $\text{DNMT activity} = (\text{sample OD} - \text{blank OD}) / [\text{protein amount (20 } \mu\text{g)} \times 2 \text{ h}] \times 1000$.

Methylated DNA Immunoprecipitation

Methylated DNA immunoprecipitation (MeDIP) assays were performed with the MeDIP Kit (ActiveMotif) following the manufacturer's instructions, as described previously [26]. Briefly, genomic DNA was extracted from P14 rat prefrontal cortex and sonicated to yield fragments ranging in size from 200 to 600 base pairs. An additional quantity of fragmented DNA equivalent to 10 % of the DNA being used in the IP reaction was saved as input DNA. The double-strand DNA fragments were denatured at 95 °C to produce single-strand DNA, and a 5-mC antibody was then used to immunoprecipitate DNA containing 5-mC. Input DNA was heat denatured and cooled following the same conditions. Both the 5-mC-enriched DNA and the input DNA were then purified with phenol/chloroform extraction and subjected to quantitative real-time PCR analysis. Sequences of primers flanking rat BDNF exons include exon VI [27], forward 5' -ACTCCCCGGCTTGGAGAAGGAAACC and reverse 5' -TCAGGGTCCACACAAAGCTCTCGGA and exon IX [28] forward 5' -CCTTCGAGAGGTCTGACGAC and reverse 5' -AGTCCGCGTCCTTATGGTTT.

Behavioral Tests

Neurobehavioral outcomes were evaluated at 3 months of age by a standard battery of neurobehavioral tests, as described previously [29, 30]. This included the tests for motor coordination and balance (rotarod test), spatial learning and memory (water maze test), general activity level (open field test), anxiety-like behaviors (zero maze test), and depression-like behaviors (forced swim test). For the rotarod test, each rat was placed on the rotarod (Rotamex-5, Columbus Instruments, Columbus, OH) that consisted of a rotating spindle 7 cm wide and had to continuously walk forward to avoid falling. Latency to fall (sec) was recorded as a measure of motor coordination and balance. For the zero maze test, the elevated zero maze test used an elevated circular track of 10 cm wide with a 100-cm outer diameter and two sets of 30-cm-high walls enclosing two opposing quadrants of the track, leaving the other two opposing quadrants open and brightly lit. The animal was placed at the midpoint of one of the open areas of the track and allowed to explore for 5 min. The percentage of time spent in the enclosed (dark) half of the maze was recorded. For the forced swim test, the rat was placed in a plastic cylinder (13.5 × 9 in), one at a time, containing about 6 in high of water (22–25 °C) for 6 min. The latency to initial immobility and total percent time in immobility were recorded. Immobility was defined as either a complete lack of movement or gradually pedaling with hind legs simply to remain afloat. For the open field test, the open field activity test involved observation for 30 min in opaque opentopped plastic boxes (49 cm long, 35.5 cm wide, 44.5 cm tall). Total distance moved, percent time spent moving, and in the center were recorded by an overhead camera and analyzed by a computerized tracking system (Noldus EthoVision). The water maze consisted of a metal tank 110 cm in diameter, filled with non-toxic opaque water with temperature at 22 °C. Each animal underwent 10 trials per day. For each trial, rats were released into the tank at one of four release points and allowed to find the 11-cm-diameter escape platform. The platform was visible during the cued learning task and submerged during the spatial task. The trials lasted a maximum of 60 s. Each animal was recorded with an overhead camera, and the Noldus EthoVision computerized tracking system allowed for assessment of variables such as swim distance, escape latency, proximity to target, swim speed, quadrant entry, and relative angular velocity (left/right turn bias). Cued learning, assessed on day 1, was a

control task for assessing sensorimotor and motivational deficits that may affect performance during the spatial phase.

Real-Time RT-PCR

RNA was extracted from the prefrontal cortex and hippocampus of both male and female rats at 3 months of age. RNA integrity was determined by electrophoresis that revealed intact 28S, 18S, and 5S RNA bands. To avoid the possible genomic DNA contamination, the RNA preparation was treated with RNase-free DNase I (1 U/μg RNA) at 37 °C for 30 min, before it was used to determine BDNF mRNA abundance by RT-PCR using iQ5 Thermocycler (Bio-Rad, Hercules, CA), as described previously [24]. The BDNF primer used were 5' - CGACGTCCCTGGCTGACACTTTT-3' (forward) and 5' - AGTAAGGGCCCGAACATACGATTGG-3' (reverse). Real-time RT-PCR was performed in a final volume of 25 μL. Each polymerase chain reaction mixture consisted of 600 nmol/L of primers, 33 U of M-MLV reverse transcriptase (Promega, Madison, WI), and iQ SYBR Green Supermix (Bio-Rad) containing 0.625 U Taq polymerase; 400 μmol/L each of dATP, dCTP, dGTP, and dTTP; 100 mmol/L KCl; 16.6 mmol/L ammonium sulfate; 40 mmol/L Tris-HCl; 6 mmol/L MgSO₄; SYBR Green I; 20 nmol/L fluorescence; and stabilizers. The following reverse transcription-polymerase chain reaction protocol was used: 42 °C for 30 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 20 s, 56 °C for 1 min, and 72 °C for 20 s. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal reference, and serial dilutions of the positive control were performed on each plate to create a standard curve. Polymerase chain reaction was performed in triplicate, and threshold cycle numbers were averaged.

Western Immunoblotting

For BDNF and TrkB, proteins were isolated from the prefrontal cortex and hippocampus of both male and female rats at 3 months of age, and Western blots were performed as previously described [31]. Briefly, samples with equal amounts of protein were loaded onto 10% polyacrylamide gel with 0.1 % sodium dodecyl sulfate and separated by electrophoresis at 100 V for 120 min. Proteins were then transferred onto nitrocellulose membranes and probed with primary antibodies against BDNF (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) and TrkB (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), respectively. After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyperfilm. The results were analyzed with Kodak ID image analysis software. Band intensities were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For DNMT immunoblotting, extracted NE (50 μg total protein) was resolved on a 4–12 % linear gradient SDS-polyacrylamide gel (Lonza) along with 10-μL Precision Plus Molecular Weight Standard (Bio-Rad) at 100 V for 1.5 h at room temperature (RT). All resolved proteins along with molecular weight standard were then electrophoretically transferred to PVDF membrane (Millipore). After blocking, the upper half (250 to 75 kDa) of membrane was probed with primary antibody against DNMT1 (D59A4) rabbit mAb (1:500, Cell Signaling), DNMT3A (D23G1) rabbit mAb (1:500, Cell Signaling), and DNMT3B antibody NB100-56514 (1:250, Novus) in TBST+ 2.5 % milk for 16 h at 4 °C, followed by incubation with secondary antibody (1:2000) in TBST+ 2.5 %

milk for 1.5 h at RT. Finally, processed PVDF membranes were extensively washed with TBST, and DNMT1, 3a and 3b were detected by incubation with Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific) and basic blue film optimized for chemiluminescence.

Statistical Analysis

Data are expressed as mean $\pm$ SEM. Experimental number (n) represents the animals from different dams. The behavioral data were analyzed with repeated measures and two-way analysis of variance (ANOVA). Correlations between behavioral variables were determined using the Pearson's product-moment coefficient. Interactions between factors were reported if statistically significant. Other molecular studies were determined by one-way ANOVA, followed by Neuman-Keuls post hoc testing or Student's t test, where appropriate. For all comparisons, $p < 0.05$ indicated statistical significance.

Results

Neonatal 5-Aza Treatment Adversely Affects the Growth Trajectory

Newborn rats were treated with 5-Aza (1 mg/kg) via i.p. injection daily from postnatal day 1 to 3 (P1–3). As shown in Fig. 1a, 5-Aza significantly reduced body and brain weight but increased the brain to body weight ratio in both P14 male and female rats. These adverse effects persisted through 3 months of age in both genders without a catch-up compensation (Fig. 1b), suggesting that exposure to 5-Aza at neonatal stage induced an irreversible and long-lasting asymmetric growth restriction.

Neonatal 5-Aza Treatment Inhibits DNMT Activity and Decreases Global DNA Methylation in the Brain

It is well recognized that 5-Aza acts as a broad spectrum DNMT inhibitor. Thus, we evaluated the effects of 5-Aza treatment from P1 to P3 on the global methylation status in the neonatal rat brain. As shown in Fig. 2a, compared to the saline control, quantitative 5-mC ELISA revealed that 5-Aza treatment significantly reduced global DNA methylation levels in the P14 rat brain of both sexes, suggesting that 5-Aza treatment effectively altered the normal DNA methylation patterns and induced global hypomethylation in the developing brain in both male and female rats. Consistent with the finding of 5-mC ELISA, 5-Aza inhibited total DNMT activities (Fig. 2b) in the brains of both male and female rats. Although 5-Aza treatment had no significant effects on protein abundance of DNMT1 and 3a in both sexes, it decreased DNMT3b in male but not female brains (Fig. 2c).

Neonatal 5-Aza Treatment Induces Aberrant Neurobehavioral Outcomes in a Sex-Specific Manner

A series of standard neurobehavioral tests were conducted to evaluate the effects of neonatal DNA methylation inhibition on neurobehavioral phenotypes in adulthood. Behavioral domains tested included sensorimotor coordination and balance (rotarod test), spatial learning and memory (water maze test), general activity (open field test), anxiety/risk-taking behaviors (zero maze test and open field test), and depression-like behaviors (forced swim test). Of great interest, neonatal 5-Aza treatment resulted in gender-specific effects on

neurobehavioral outcomes in adult rats. As shown in Fig. 3, all groups performed similarly when the rotarod spun at a constant speed. When the task was made more difficult by accelerating the rate of spin, the performance of saline-treated males dropped significantly, and neonatal 5-Aza treatment prevented this decline. 5-Aza exposure induced no significant performance changes in female rats. However, other tests demonstrated that female rats were more sensitive to 5-Aza treatment. Saline-treated females spent significantly more time in the enclosed quadrants of the elevated zero maze than 5-Aza-treated females, which performed similarly to males from both treatment groups (Fig. 4a). In the forced swim test (Fig. 4b), 5-Aza-treated female rats demonstrated longer latency to immobility and significantly less time in immobility, suggesting an anti-depressant-like effect of neonatal 5-Aza treatment that was especially pronounced in females. In addition, although there were no significant changes in general activity levels in the open field test (Fig. 4c), female rats treated with 5-Aza spent significantly more time in the center (rather than the periphery), which demonstrates less anxiety and/or increased risk-taking behaviors, which is consistent with findings in the zero maze test. Interestingly, 5-Aza significantly improved spatial learning capacity of female rats in the water maze test. As indicated in Fig. 5, compared with the saline control, 5-Aza-treated female rats found the escape platform more quickly (Fig. 5a) and efficiently (Fig. 5c) during learning trials. Additionally, although 5-Aza females did not cross former platform locations more often during the probe trials (Fig. 5d), they spent more time searching in the target quadrants (Fig. 5b), suggesting improved spatial memory.

Neonatal 5-Aza Treatment Abrogates Gender Differences in Neurobehavioral Phenotypes in Adult Rats

More importantly, we observed apparent sexually dimorphic differences in neurobehavioral performance in saline-treated control animals. Neonatal 5-Aza treatment effectively eliminated such sexual differences in adulthood. As shown in the rotarod test (Fig. 3), female rats demonstrated better performance in the slow- and fast-accelerating tests than male rats, while 5-Aza treatment removed these differences. Both in the zero maze test (Fig. 4a) and open field test (Fig. 4c), female rats spent more time in the enclosed quadrants of the zero maze and significantly less time in center of the open field compared to males, suggesting higher levels of anxiety and/or reduced tendencies for risky exploratory behaviors in female rats. However, no significant differences were observed between male and female rats with neonatal 5-Aza treatment. In addition, in the water maze test, male rats demonstrated better spatial learning performance compared to females (Fig. 5a–c). However, neonatal 5-Aza treatment significantly improved spatial learning ability in female rats and abolished such sexual differences. These findings indicate that neonatal 5-Aza treatment may disrupt sexually dimorphic differentiation of neurobehavioral phenotypes later in life.

Neonatal 5-Aza Treatment Decreases BDNF Promoter Methylation and Increases Brain Expression of BDNF in a Sex-Specific Manner

It is well documented that BDNF-TrkB signaling in the brain plays a critical role in the regulation of cognition, emotion, and behavior. We thus evaluated the effects of neonatal 5-Aza treatment on the expression profiles of BDNF-TrkB in the mature rat brain to reveal its linkage to the observed behavioral outcomes. 5-Aza treatment in newborn rats resulted in an enduring and sex-specific effect on BDNF-TrkB expression in adult rat brains. As shown in

Fig. 6, 5-Aza selectively up-regulated BDNF mRNA levels in the prefrontal cortex (Fig. 6a) and hippocampus (Fig. 6b) in female rats, with no significant effect on BDNF mRNA in male animals. We further determined whether 5-Aza treatment specifically altered methylation levels at the BDNF gene promoters in the neonatal rat brain. As shown in Fig. 7, compared to the saline control, 5-Aza treatment caused a sexdependent decrease by 94 % in methylation levels of the BDNF exon IX promoter in female brains. Unlike exon IX, methylation of BDNF exon VI was not significantly changed in either male or female brains. Consistent with the increased mRNA abundance, Western blotting indicated that 5-Aza-treated female rats demonstrated greater expression of pro-BDNF and mature-BDNF protein abundance in both the prefrontal cortex (Fig. 8b) and hippocampus (Fig. 8d). In contrast, no significant increase in pro-BDNF or mature-BDNF protein was observed in 5-Aza-treated male animals (Fig. 8a, c). Of interest, 5-Aza also induced a significant increase in TrkB protein abundance in the prefrontal cortex, but not in the hippocampus, in both male and female rats (Fig. 8).

Discussion

The present study reveals a novel mechanism of DNA methylation in the sex dimorphism of brain development and neurobehavioral phenotypes later in life. Thus, inhibition of DNA methylation during a critical window of the brain development resulted in aberrant neurobehavioral outcomes in a sex-dependent manner. Of importance, inhibition of DNA methylation in the developing brain disrupted pre-programmed sexually dimorphic differences in neurobehavioral phenotypes in adulthood. In addition, brain DNA hypomethylation in the early developmental stage caused reprogrammed expression patterns of BDNF-TrkB signaling in the mature brain, which may contribute to aberrant sex-specific neurobehavioral outcomes resulting from brain hypomethylation.

Sexually dimorphic differentiation of the brain and behavior has been well documented, albeit the underlying mechanisms remain poorly understood. It is generally accepted that testosterone, and its metabolites estradiol and dihydrotestosterone, are crucial for organizing some of the most salient sex differences in the brain and behavior [32, 33]. Steroid receptors, including estrogen receptor α (ER α), ER β , androgen receptor (AR), and progesterone receptor (PR), have also been implicated in sexually differentiating the brain during the sensitive periods of behavioral development [19, 34]. Early exposure to steroids exerts enduring effects, and most brain sex differences are established during a critical developmental window, usually from embryonic day 18 to postnatal day 10 in rodents [35, 36]. Steroid hormones interact with their respective receptors in the developing brain, producing sustained sexual differences in cell number, migration, phenotype, and highly sexual dimorphic brain regions, as well as programming of dimorphic behaviors in later life. It is possible that epigenetic regulations contribute to some of these sexually dimorphic outcomes. In fact, emerging evidence has revealed gender differences in DNA methylation patterns at promoter regions of steroid receptors, methyl-binding proteins (i.e., MeCP2), and corepressor proteins (i.e., NCoR) during a sensitive period of the brain development [19, 37, 38], suggesting a potential role for DNA methylation in the sexually dimorphic differentiation of the brain and behavior. Jessen et al. reported that neonatal female rodents expressed significantly higher levels of MeCP2, NCoR, and DNMT3a than males within

specific brain regions, including the amygdale and hypothalamus [39]. McCarthy et al. reported that treatment of newborn male rats with DNMT inhibitors reduced DNA methylation and altered gene expression profiles, leading to masculinization of the synaptic patterning in the preoptic area, a highly sexually dimorphic region, and sexual behaviors [22]. Furthermore, these studies showed that masculinization required demethylation and that feminization was a repression of masculinization via DNA methylation, suggesting a critical role of DNA methylation in brain sexual differentiation and feminization being maintained by DNA methylation [22, 23].

In the present study, we observed apparent sexually dimorphic differences in several domains of neurobehavioral function in the control animals. Compared with male rats, female animals appeared to present with higher level of anxiety, lower ability in spatial learning, but better sensorimotor coordination and balance. 5-Aza administration in newborns significantly reduced brain global DNA methylation levels and inhibited total DNMT activities in both male and female rats and effectively abolished the sex differences in neurobehavioral function in adult rats of 3 months old. This finding provides novel and exciting evidence in supporting the notion that brain DNA methylation profiles early in life are associated with the establishment and maintenance of sexually dimorphic differentiation of the brain and behavior later in life [22, 23]. However, distinct from previous studies that focused on investigating primitive reproductive behaviors, our study mainly explored the potential programming effects of DNA methylation in the setting of more complex neuropsychiatric activities, including anxiety, depression, general activity, spatial learning, and memory. For the first time, we revealed a correlation between inhibition of global DNA methylation in the developing brain and disruption of pre-programmed sexually dimorphic neurobehavioral phenotypes in adulthood. This finding is important because it may contribute to further elucidating the pathogenesis in sexually dimorphic risk or resilience for developing neurological and mental health disorders, given the well-known sexual bias in the prevalence and symptomology of mental disorders [20, 21, 39]. In contrast to previous studies, we did not observe significant sex differences of the global DNA methylation level in the neonatal rat brain, possibly due to the selected brain developmental stage, brain tissue collection, or the sensitivity of experimental technique in these studies. Nonetheless, a question remains to be determined is the implication of steroid hormones and their respective receptors in the specific brain regions that might be regulated by DNA methylation and involved in the sex pathogenesis observed in the present study.

It has been demonstrated that BDNF-TrkB signaling is an important regulator of neural survival, development, synaptic plasticity, brain function, and a wide range of behavioral phenotypes [40–43]. BDNF is widely expressed in multiple brain regions, including the frontal cortex, hippocampus, amygdale, corpus callosum, basal ganglia, thalamus, brainstem, pons, and cerebellum [44, 45]. Accumulating evidence has suggested that BDNF gene transcription is highly dependent on DNA methylation status at its promoter region, which is also clearly associated with the pathophysiology of neuropsychiatric disorders [15, 27, 46–48]. The rat BDNF gene is located on the chromosome 3, which consists of nine 5′ non-coding exons, each linked to individual promoter regions, and a 3′ coding exon (IX), which codes for the BDNF pre-protein (pro-BDNF) [49, 50]. The neuronal activity-dependent regulation of specific promoter regions within the BDNF gene dictates spatial and temporal

expressions of specific BDNF transcript isoforms [28]. After translation, the produced pro-BDNF protein is further cleaved into a mature form of BDNF via diverse enzymatic cleavage [51], which then binds to TrkB and regulates neural differentiation and survival, neurite outgrowth, and synaptic plasticity [52]. In the present study, neonatal 5-Aza treatment not only induced reduction of methylation levels at BDNF promoter in the developing brain but also significantly increased expression levels of BDNF mRNA in the adult brain, further supporting the notion of promoter-DNA-methylation-dependent BDNF gene expression in the central nervous system. Interestingly, 5-Aza-induced effects were predominantly observed in female rats. Although it is not clear how this sex-specific effect is induced by 5-Aza treatment at present, it is possible that steroid hormones and their receptors may be differentially involved. Consistently, Western blotting revealed a significant increase in both pro-BDNF and mature-BDNF protein abundance in the prefrontal cortex and hippocampus in female rats treated with 5-Aza. Given well-documented positive effects of BDNF in the regulation of emotion, mood, and cognition [15, 46, 47], it may serve as one of the potential mediators underlying the DNA hypomethylation-induced sex-specific neurobehavioral outcomes observed in the present study. In addition, we observed that 5-Aza treatment significantly up-regulated TrkB protein abundance in the prefrontal cortex of both male and female rats, suggesting that DNA hypomethylation also regulates TrkB expression in a specific brain region. In general, our study confers a clear correlation between aberrant BDNF profiles induced by DNA hypomethylation early in life and the sex-specific neurobehavioral outcomes in adulthood. It is intriguing that female rats with 5-Aza exposure appeared to demonstrate better performance in neurobehavioral tests, including lower anxiety, less depression, and higher ability in spatial learning, in comparison to its saline control. How to define the significance of such abnormally improved neurobehavioral phenotypes in the context of clinics is an interesting challenge, which may yield novel elucidation of sexually dimorphic risk or resilience for developing neuropsychiatric disorders later in life or confer promising intervention for these disabling mental disorders.

Several questions remain to be answered. 5-Aza acts as a wide spectrum DNA methylation inhibitor, lacking gene-specific effects. Thus, the present study mainly revealed the effects of global DNA hypomethylation in the developing brain on the neurobehavioral development in adulthood. It is possible that changes in DNA methylation patterns in the brain are highly gene specific and tightly temporal-spatial dependent, all of which are interacted and contribute to the development of complex neurobehavioral phenotypes, including sexually dimorphic differences. Secondly, the interaction between DNA methylation, steroid hormones and their respective receptors, and their potential roles in programming of sexually dimorphic effects in neurobehavioral development remain to be explored. Not only aberrant DNA methylation profiles in the brain, given the adverse effects of 5-Aza on overall growth in this study, but it is also conceivable that neonatal 5-Aza treatment may alter or disrupt sexual maturity or reproductive status in animals, which more or less contributes to abnormal neurobehavioral phenotypes in adulthood. Furthermore, in addition to BDNF-TrkB signaling, other molecules and signaling pathways including glucocorticoids, corticotropin-releasing hormone, and the hypothalamic-pituitary-adrenal axis are also implicated in the development and regulation of complex behaviors. It will be worthwhile to

further explore the possible effects of 5-Aza-induced brain DNA hypomethylation early in life on these pathways and their relationship to behavioral development in later life.

In conclusion, our present study revealed a simple, but important, observation; DNA methylation in the brain early in life plays an essential role in the programming and maintenance of sexually dimorphic differentiation of the brain and neurobehavioral phenotypes later in life. The finding not only provides novel evidence to elucidate the mechanisms underpinning gender differences in behavioral development at the epigenetic level but also may contribute to unveiling the complex pathogenesis of sexually dimorphic risk or resilience for developing neuropsychiatric disorders later in life. In addition, our findings further revealed the programming effects of 5-Aza-induced DNA hypomethylation in altering BDNF-TrkB expression in the brain, suggesting a possible molecular link and a novel avenue of therapeutic target involving epigenetic modifications for certain refractory debilitating neuropsychiatric disorders.

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Fig. 1. Effect of neonatal 5-Aza treatment on body weight, brain weight, and the brain to body weight ratio. 5-Aza (1 mg/kg BW, i.p.) or saline was administered to newborn rats daily from postnatal day 1 to 3. Body weight and brain weight were determined at P14 (a) and 3 months (b) of age, respectively. Data are means $\pm$ SEM. $n = 8-12$. * $p < 0.05$, 5-Aza versus saline control

Fig. 2.

Effect of neonatal 5-Aza treatment on global DNA methylation, DNMT activity, and DNMT protein abundance in the neonatal rat brain. 5-Aza (1 mg/kg BW, i.p.) or saline was administered to newborn rats daily from postnatal day 1 to 3. Brain DNA methylation levels (a), DNMT activity (b), and DNMT1, 3a and 3b protein abundance (c) were determined in P14 pups. Data are means $\pm$ SEM. $n = 5-6$. * $p < 0.05$, 5-Aza versus saline control

Fig. 3.

Effect of neonatal 5-Aza treatment on rotarod test in adult rats. 5-Aza (1 mg/kg BW, i.p.) or saline was administered to newborn rats daily from postnatal day 1 to 3. The rotarod test was conducted in adult rats at 3 months of age. Data are means $\pm$ SEM. $n = 10$. * $p < 0.05$, 5-Aza versus saline control; # $p < 0.05$, female versus male in saline control

Fig. 4.

Effect of neonatal 5-Aza treatment on zero maze test, forced swim test, and open field test in adult rats. 5-Aza (1 mg/kg BW, i.p.) or saline was administered to newborn rats daily from postnatal day 1 to 3. Zero maze test (a), forced swim test (b), and open field test (c) were conducted in adult rats at 3 months of age. Data are means $\pm$ SEM. $n = 10$. * $p < 0.05$, 5-Aza versus saline control; # $p < 0.05$, female versus male in saline control

Fig. 5.

Effect of neonatal 5-Aza treatment on water maze test in adult rats. 5-Aza (1 mg/kg BW, i.p.) or saline was administered to newborn rats daily from postnatal day 1 to 3. Water maze test was conducted in adult rats at 3 months of age. **a** Escape latency, **b** total duration in quadrant, **c** distance to platform average, and **d** target entry probe. Data are means $\pm$ SEM. $n = 10$. * $p < 0.05$, 5-Aza versus saline control; # $p < 0.05$, female versus male in saline control

Fig. 6. Effect of neonatal 5-Aza treatment on BDNF mRNA abundance in adult brain. 5-Aza (1 mg/kg BW, i.p.) or saline was administered to newborn rats daily from postnatal day 1 to 3. BDNF mRNA abundance was determined in the prefrontal cortex (**a**) and hippocampus (**b**) in adult rats at 3 months of age. Data are means $\pm$ SEM. $n = 5-6$. * $p < 0.05$, 5-Aza versus saline control

Fig. 7.

Effect of neonatal 5-Aza treatment on BDNF promoter methylation in neonatal rat brain. 5-Aza (1 mg/kg BW, i.p.) or saline was administered to newborn rats daily from postnatal day 1 to 3. Methylation levels at BDNF promoter of exons VI and IX in the prefrontal cortex of P14 rats were determined with MeDIP. Data are expressed as percent of saline control and are means $\pm$ SEM. $n = 5$. * $p < 0.05$, 5-Aza versus saline control

Fig. 8.

Effect of neonatal 5-Aza treatment on protein abundance of BDNF and TrkB in adult brain. 5-Aza (1 mg/kg BW, i.p.) or saline was administered to newborn rats daily from postnatal day 1 to 3. Protein abundance of pro-BDNF, mature BDNF, and TrkB was determined in the prefrontal cortex of male (a) and female (b) and in the hippocampus of male (c) and female (d) adult rats at 3 months of age. Data are means $\pm$ SEM. $n = 5$ to 6. * $p < 0.05$, 5-Aza versus saline control