Intrauterine Position Affects Estrogen Receptor α Expression in the Ventromedial Nucleus of the Hypothalamus via Promoter DNA Methylation

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There is well-established evidence in many mammalian species for effects of the intrauterine position (IUP) (the sex-specific positioning of the embryo) on postnatal brain function and behavior. We found that the IUP affects estrogen receptor (ER)α expression in adult female rats in the ventrolateral region of the hypothalamic ventromedial nucleus (vlVMH), which is associated with sexual behavior. The ERα expression level in the vlVMH was higher in females that developed in utero between two male siblings (2M females) than in those that developed between female siblings (2F females). We also found that the cytosine methylation status across the ERα promoter in the vlVMH was affected by the IUP, with greater methylation in 2F females. These findings showed a negative correlation between ERα expression levels in the vlVMH and methylation frequency in the ERα promoter. This suggests that genomic methylation sustains the effect of the fetal IUP on ERα expression in the vlVMH. (Endocrinology 151: 5775–5781, 2010)

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nteruterine programming is an imprinting process in which individual phenotypes are permanently modified by environmental conditions during development (1–4). The effect of the intrauterine position (IUP) provides a natural model system for examining the programming effects of the intrauterine environment, as determined by the sex of neighboring fetuses in polytocous animals (5–8) (Fig. 1). Several lines of evidence show a programming effect of the IUP on the development of female phenotypes. Among adult phenotypes known to be sexually differentiated, the IUP influences the morphology of external genitalia, with greater masculinization in females that developed in utero between two male siblings (2M females). This masculinizing effect results from fetal androgenic exposure due to the proximity of male siblings in utero (6–9).

During the late gestational period, large amounts of testosterone are secreted by the male fetal testes. A metabolite, estradiol, then causes morphological and phenotypical masculinization, whereas in the absence of these hormones mammalian fetuses develop into phenotypic females (10–12). Females exposed to higher levels of testosterone, estradiol, or environmental androgenic/estrogenic chemicals in utero display a disrupted estrous cycle and masculinized behavior during adulthood (5, 13–16). Courtships and sexual behavior are also determined by fetal androgenic exposure in rats (11, 18, 19). The influence of the IUP on these behavioral characteristics is evident: 2M females show greater aggressiveness and less sexual receptivity than females that developed in utero between two female siblings (2F females) (6–8). However, the neurological basis of these IUP-related differences in behavior is unclear.

Estrogenic activities in the ventrolateral region of the ventromedial nucleus of the hypothalamus (vlVMH) promote female aggression and sexual behavior and are primarily mediated by estrogen receptor (ER)α (20, 21). Because the extent of these behavioral characteristics is thought to be influenced by the expression levels of ERα (14, 19), we hypothesized that there is a correlation between IUP-related individual variation in behavior and sex. The Endocrine Society. Downloaded from press.endocrine.org by [individualUser.displayName] on 13 January 2015 at 15:10 For personal use only. No other uses without permission. All rights reserved.
differential ERα expression in the vlVMH. To investigate this, we compared ERα expression levels in the vlVMH of 2M and 2F female offspring and found them to differ. This was thought to be the result of differences in hormonal exposure depending on the IUP during the hormonally sensitive period of development. How, then, do the IUP-induced hormonal consequences of different intrauterine environments translate into the adult characteristics? One possibility for the causal route from early androgenic exposure to later ERα expression is genomic modification by DNA methylation (1, 4, 22).

The functional contribution of genomic methylation to development and imprinting phenomena is becoming increasingly evident, and methylation may serve as a memory of early regulatory events (2, 23, 24). Genomic methylation, in which the cytosine at CpG dinucleotides is methylated by methyltransferases, is known to moderate transcriptional activity. Cytosine methylation induces transcriptional silencing of a targeted genome by formation of inactive chromatin structure and interference with transcription factor binding (1, 23, 24). Methylation patterns can be retained over a lifetime (1, 4), and a significant contribution of methylation to the persistence of gene expression is becoming increasingly evident in imprinting phenomena (2, 23) and may serve as a memory of early regulatory events. In the current study, we investigated the involvement of cytosine methylation of the ERα promoter in the vlVMH on IUP programming of female phenotypes.

Materials and Methods

Animals

Pregnant Wistar rats were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). All fetuses were delivered by Cesarean section under anesthesia (a lethal dose of sodium pentobarbital, 200 mg/kg body weight ip) on d 21 of gestation. Fetal sexing was performed on the basis of the anogenital distance (8) (Fig. 1), and the IUP of each fetus was marked using a toe-clipping pattern (25). All offspring were cross-fostered to a foster mother that had delivered vaginally within the previous 24 h. The pups were weaned on d 30 postpartum and housed in a cage with littermates of the same sex until adulthood. At 10 wk of age females were followed with vaginal smears and used for experiments when at the estrus stage of the estrous cycle (n = 71 from 23 litters). All animals were housed under a 12-h light, 12-h dark cycle and given food and water ad libitum. Animal care, maintenance, and surgery were performed in accordance with the Rules and Regulations for Animal Research, Kyoto Prefectural University of Medicine.

Tissue preparations

For immunohistochemistry, 2M (n = 5) and 2F (n = 6) female offspring from five litters were killed as described above and perfused intracardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were removed and postfixed, stored at 4 C in 0.1 M PBS containing 25% sucrose, and then sectioned at 20-μm thickness using a cryostat (CM3050, Leica, Solms, Germany). Sections were immunostained with rabbit anti-ERα antiserum (MC-20, 1:2000 dilution in Tris-buffered saline with Tween-20 (TBST); Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and mounted on glass slides (26).

Female offspring were killed at 10 wk of age for Western blotting (2M females, n = 5; 2F females, n = 6; 2M males, n = 8; and 2F males, n = 5 from seven litters), real-time PCR (2M, n = 4; 2F, n = 6; and 1M females: females that were developed between one male and female, n = 6 from six litters), and sodium bisulfite mapping (2M, n = 6; and 2F, n = 6 from six litters). Whole brains were removed under anesthesia (lethal dose of sodium pentobarbital, 150 mg/kg body weight ip and 2% isoflurane). To obtain VMH tissue selectively, the brains were cut into 500-μm thick coronal sections using a microvibratome (D.S.K. Microslicer DTK-300W; Dosaka EM, Kyoto, Japan). Two sections containing the entire VMH region were mounted on glass, and the ventrolateral part of the VMH (vlVMH) was dissected bilaterally using a stainless steel needle (inner diameter 900 μm) under a stereomicroscope (Fig. 2). Rat brain maps (27) and preliminary Nissl staining and ERα immunostaining were used to confirm identification of the vlVMH region. All dissected tissues were snap frozen in liquid nitrogen.

Western blot analysis

Tissue from the vlVMH region from 2M (n = 5), 2F (n = 6) females, 2M males (n = 8), and 2F males (n = 5) were lysed with 30 μl of sample buffer. After boiling for 5 min, the lysates (10 μl for ERα detection, 4 μl for tubulin detection) were subjected to 7.5% SDS-PAGE. Samples were electroblotted onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) using a semidry blotting apparatus (Bio-Rad Laboratories, Hercules, CA). The blotted membranes were blocked with 1.5% skim milk TBST at 4 C for 20 min, and then incubated with anti-ERα antibodies and antitubulin antibodies (MC-20, 1:30,000 dilution in TBST; Sigma Japan Ltd., Tokyo, Japan) at 4 C for 12 h. Blots were washed three times with TBST and incubated with alkaline phosphatase-conjugated anti-IgG secondary antibody (1:1000 dilution in TBST; Chemicon, Temecula, CA) for 2 h at room temperature. After being washed three times with TBST, the blots were visualized using an nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.
Quantitative real-time PCR
Total RNA was extracted using an RNeasy Micro Kit (QIAGEN, Hilden, Germany) from each dissected vLVVMH tissue sample from 2M (n = 4), 1M (n = 6) females and 2F females (n = 6) and reverse transcribed using ReverTra Ace (Toyobo, Osaka, Japan) and random hexamers according to the manufacturer’s guidelines. Gene expression was assessed by quantitative real-time PCR using a Light Cycler 480 II with Universal Probe Library assays (Roche Diagnostics, Mannheim, Germany). Reactions were performed in duplicate in 96-well plates for 55 amplification cycles (95 C, 10 sec; 60 C, 10 sec; 72 C, 10 sec) in a 10-μl reaction volume. Primers were designed using the Roche Universal Probe Library Assay Design Center (www.universalprobelibrary.com): ERα forward primer: AGG CTG CAA GGC TTT CTT TA; ERα reverse primer: TCT TAT CTA TGG TGC ATT GG; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward primer: ACA ACT TTT GCA TCG TGG A; GAPDH reverse primer: CCT CTG AGT GGC AGT GAT GG. The carboxyfluorescein-labeled probe (Universal Probe Library, Roche Diagnostics) numbers were 130 for ERα and 114 for GAPDH. For quantification of changes in gene expression, the comparative C_p (threshold cycle number) method was applied to calculate the relative fold changes normalized against GAPDH (% GAPDH).

Sodium bisulfite mapping
Total DNA was collected using NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) from each dissected vLVVMH tissue sample from 2M (n = 6) and 2F (n = 6) females according to the protocol provided in the kit. DNA solution was concentrated by QIAquick PCR Purification Kit (QIAGEN). The DNA was first reacted with sodium bisulfate and incubated (12 h, 50 C) to convert unmethylated cytosine to uracil while leaving 5-methylcytosine unchanged (EZ DNA Methylation Kit; Zymo Research Corp., Orange, CA). All the procedure for Sodium Bisulfite conversion followed the protocol providing in the kit (Zymo Research Corp.). Isolation and amplification of bisulfite-treated genomic DNA were performed using strand-specific PCR with primers targeting the bisulfite-converted DNA of exon 1b in the ERα promoter region (GenBank accession no. × 98236) (outside, forward: 1874-TAG TAT ATT TGA TTG TTA TTT TAT-1898; reverse: 2377-TCT CAA ACC ACTCAA AC-2358) (23). The thermocycler protocol followed an initial denaturation cycle (5 min, 95 C); 30 cycles of denaturation (1 min, 95 C), annealing (2 min, 30 sec, 54 C), and extension (1 min, 72 C); and a final extension cycle (5 min, 72 C) terminating at 4 C. The PCR product (504 bp) was used for subsequent PCRs with nested primers (forward: 1899-TTT ATT TGT GGT TTA TTA TTA.TAGATA ATA TTT-1922; reverse: 2377-ACA AAA AAA AAA TAA TCA AAA AAC-CAC-2334). The nested PCR product (479 bp) was then subcloned (TAwgeron Clone, Toyobo) and transformed, and 10 different clones per plate were miniprepared. Only plasmids containing the exon 1b ERα promoter region (10 positive plasmids per animal) were collected and sequenced. The methylation pattern of individual CpG sites across exon 1b was averaged per animal per group, and the percentage of methylated cytosine residues (mean ± SEM) was compared between 2M and 2F females.

Statistical analysis
One-way ANOVA and Student’s t test were used to analyze data from immunohistochemistry and quantitative real-time PCR. One-way ANOVA followed by Tukey-Kramer post hoc
analysis (multiple tests) were used for Western blotting analysis. Two-way repeated ANOVA followed by Tukey-Kramer post hoc analysis were used for sodium bisulfite mapping data to analyze the IUP effect on cit-specific cytosine methylation. Overall average of CpG methylation level was analyzed using one-way ANOVA and Student’s t test. In all statistical analyses, values are shown as the group mean ± sem, and P < 0.05 was considered statistically significant.

Results

IUP effects on ERα expression in the VMH region

During observation of ERα-ir in rat brain sections from 2M and 2F adult females, we noticed individual variations in ERα immunostaining patterns in the VMH region. A preliminary examination suggested that the staining intensity was related to IUP during development, because staining intensity was consistently enhanced in 2M females, but moderate in 2F females (Fig. 3A). To quantify this apparent difference in ERα staining intensity, we compared ERα protein levels between the two groups using Western blotting. Because ERα is mostly expressed within the vlVMH (20, 29, 30), we selectively dissected the vlVMH for analysis (Fig. 2). Quantitative Western blotting demonstrated that the ERα protein level in the vlVMH was significantly higher in 2M females than in 2F females, indicating a significant effect of IUP (Fig. 3, B and C). Among the sexes, ERα protein level in the vlVMH region was maximum in 2M females compared with 2M and 2F males, being significantly different (Fig. 3C). ERα-ir neurons were counted to investigate whether the IUP also affects their numbers in the vlVMH, but there was no difference in these counts for 2M and 2F females (Fig. 3D). These results show that the enhanced staining intensity in ERα-expressing neurons was due to elevated ERα expression in 2M females, and not to the number or distribution of ERα-ir neurons. We also quantified the ERα mRNA level in each group. Quantitative real-time PCR revealed different levels of mRNA for vlVMH ERα between 2M and 2F females, with significantly higher levels in 2M females (Fig. 4). These results show that the reduced ERα expression in the vlVMH of 2F females is due to lower transcriptional activity of the ERα gene, indicating IUP-related regulation of ERα expression. Additionally, we investigated the mRNA level for vlVMH ERα in 1M females (female siblings that developed in utero between male and female). ERα mRNA level in 1M female vlVMH was intermediate between 2M and 2F females.

Sodium bisulfite mapping

The difference in the ERα protein level in the vlVMH region between 2M and 2F adult females suggests early environmental regulation of ERα gene expression (Fig. 3, B and C). To investigate the mechanism mediating this long-lasting regulation of gene expression, we examined cytosine methylation across exon 1b in the ER promoter in the vlVMH of 2M and 2F adult females. The exon 1b promoter region is thought to play a major role in transcriptional regulation of ERα in rat brain and contains 17 CpG sites within 490 bp (23). Sodium bisulfite mapping
has been used for rapid detection of methylation at individual CpG sites. Bisulfite genomic sequencing revealed different methylation patterns in 2M and 2F females, with eight of 17 CpG sites within exon 1b of the ER promoter more frequently methylated in 2F females than in 2M females (Fig. 5A).

The average CpG methylation shows significant difference between 2M females and 2F females (Fig. 5B).

**Discussion**

In many species, including humans, the presence of testicular steroids in early life largely determines adult sexual phenotypes (11, 19, 31). The coincidence of sexual phenotypes and genotypes occurs as a consequence of hormonal programming of a broad range of phenotypes (12, 14). Normally, adult neuroendocrine and behavioral characteristics correspond to those expected for the genetically determined sex; however, changes in early hormonal status can cause development in the opposite direction. Similarly, sexual phenotypic variations among adults of the same sex can also occur as a result of varying degrees of early hormonal exposure during the hormonally sensitive period of development (4, 9, 11).

In polytocous animals, the random IUP of female fetuses is an important variable (Fig. 1) that significantly affects the hormonal environment during intrauterine development due to testicular hormonal diffusion from male siblings (8). Because steroid hormones are small lipophilic molecules that easily cross the blood-brain barrier, sex steroids from the male fetal gonads can diffuse to adjacent females in utero (5). During the late gestational period, both the circulating and intracerebral concentrations of testosterone are elevated in 2M fetuses compared with 2F fetuses (6, 8). The anogenital distance, which is lengthened by early exposure to androgens and serves as a sensitive biomarker for early androgenic activity, is lengthened in 2M females. Regarding behavioral phenotypes, 2M females show greater aggressiveness and lower sexual receptivity than 2F females, indicating that the subtle increase in exposure to sex steroids resulting from proximity to male fetal siblings is sufficient to cause morphological and physiological changes in female offspring (6, 8).

We have previously shown that the IUP affects the individual morphology of the sexual dimorphic nucleus of the preoptic area (SDN-POA) in rats (25). Early sex-steroid exposure results in an enlarged SDN-POA; thus, male rats have a larger SDN-POA than female rats and more neuronal nuclei. We also observed IUP-related differences in the same sex, with the volume of the SDN-POA being significantly larger in 2M males compared with 2F males (26).

In the present study, we found that the ERα expression level in the vlVMH was significantly increased in 2M females compared with 2F females, indicating early regulation of sexual phenotypes of ERα expression. The molecular mechanism through which the programming effect of sex steroids during intrauterine development persists into adulthood is of particular interest. Recent evidence indicates epigenetic regulation is crucial in early regulatory phenomena in postnatal gene expression (1, 4, 17). The process of sexual differentiation of the neural network in
the rat POA is an example in which epigenetic regulation plays an important role in masculinization of the developing male POA (22). In this process, early testicular hormone exposure can result in methylation marks on the genome, causing lasting masculinization of the POA.

This epigenetic regulatory mechanism is becoming established as a potent mediator of several imprinting parameters, such as prenatal and postnatal nutritional and hormonal environments (3, 17). Champagne et al. (23) reported that individual behavioral characteristics of maternal care in female rats can be programmed by neonatal events, most likely being mediated via epigenetic gene modification and in particular by cytosine methylation across the genome. Individual female maternal-care phenotypes were shown to depend on whether animals received higher or lower licking and grooming (LG) maternal care (high LG or low LG) during postnatal development (24). Furthermore, the expression of ERα in the medial POA is also programmed (18, 23). Females that received high LG exhibited elevated ERα expression and high LG maternal care in adulthood, whereas females that received low LG exhibited moderate ERα expression and low LG maternal care. Cytosine methylation patterns across the ERα promoter region also differed significantly among female offspring: CpG sites within the ERα promoter were more frequently methylated in the offspring of low-LG dams than high-LG dams (24).

Given these findings, the programming effects induced by intrauterine proximity to male siblings (the IUP phenomenon) may be mediated by epigenetic gene modification. We observed the cytosine methylation status across the ERα Ib promoter region in the vlVMH in 2M and 2F female offspring and found differences in methylation patterns: CpG sites across the ERα Ib promoter region in the vlVMH were more densely methylated in 2F females than in 2M females. In general, methylation density within promoter regions in the genome is inversely related to transcriptional activity. Cytosine methylation induces transcriptional silencing of target genes by formation of an inactive chromatin structure and by interfering with transcription factor binding to the promoter (1, 3, 23). If the ERα Ib promoter region containing transcription factor-binding sites is essential for enhancement of basal transcriptional activity (23), methylation of this region may interfere with the recruitment and binding of transcription factors, and consequently result in reduced gene expression (22, 24). Alteration of the DNA methylation status of the ERα Ib promoter may not explain all aspects of the behavioral differences between 2M and 2F individuals, but these findings indicate that differences in expression of several or multiple genes generated by alterations of DNA methylation status can be caused by variable factors including IUP during brain development can result in individual behavioral differences.

The present study suggests that genomic methylation contributes to the IUP phenomenon in maintaining the memory of hormonal exposure during fetal life to adulthood, with a persistent effect on gene expression. Because the developing genome is labile and exclusively sensitive to several environmental stimuli (3), elevated exposure to sex steroids during a hormonally sensitive period could be significant and may be imprinted on the genome as epigenetic information. Thus, DNA methylation may be the genomic modification that serves to characterize individual features in response to subtle changes in the environment in which individual animals develop, resulting in a wide variety of phenotypes.

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