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Maternal Care Associated with Methylation of the Estrogen Receptor-α1b Promoter and Estrogen Receptor-α Expression in the Medial Preoptic Area of Female Offspring

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Variations in maternal behavior are associated with differences in estrogen receptor (ER)-α expression in the medial preoptic area (MPOA) and are transmitted across generations such that, as adults, the female offspring of mothers that exhibit increased pup licking/grooming (LG) over the first week postpartum (*i.e.* high LG mothers) show increased ERα expression in the MPOA and are themselves high LG mothers. In the present studies, cross-fostering confirmed an association between maternal care and ERα expression in the MPOA; the biological offspring of low LG mothers fostered at birth to high LG dams show increased ERα expression in the MPOA. Cross-fostering the biological offspring of high LG mothers to low LG dams produces the opposite effect. We examined whether the maternal programing of ERα expression is associated with differences in methylation of the relevant ERα promoter. Levels of cytosine methylation across the ERα1b promoter were significantly elevated in the adult offspring of low, compared with high, LG mothers. Differentially methylated regions included a signal transducer and activator of transcription (Stat)5 binding site and the results of chromatin immunoprecipitation assays revealed decreased Stat5b binding to the ERα1b promoter in the adult offspring of low, compared with high, LG mothers. Finally, we found increased Stat5b levels in the MPOA of neonates reared by high, compared with low, LG mothers. These findings suggest that maternal care is associated with cytosine methylation of the ERα1b promoter, providing a potential mechanism for the programming of individual differences in ERα expression and maternal behavior in the female offspring. (*Endocrinology* 147: 2909–2915, 2006)

VARIATIONS IN MATERNAL care in the rat have profound consequences for the development of offspring. Offspring of mothers that display high levels of pup licking/grooming (LG; *i.e.* high LG mothers) exhibit more modest hypothalamic-pituitary-adrenal responses to stress, enhanced cognitive ability in tests of hippocampal function, and altered reproductive behavior (1–5). Variations in maternal care are transmitted across generations, such that the lactating adult offspring of high LG mothers are themselves high in pup LG, whereas the offspring of low LG mothers exhibit decreased levels of pup LG (4, 5). In the rat, central oxytocin (OT) receptors are obligatory for the expression of maternal behavior (6–8), and variations in OT receptor levels in critical brain regions, such as the medial preoptic area of the hypothalamus (MPOA), are functionally linked to differences in maternal care (5). OT receptor binding in the MPOA is increased in high compared with low LG mothers (5, 9). Central infusion of a selective OT antagonist on postpartum d 3 reduces LG behavior in high LG dams and abolishes group differences in LG, without altering the total amount of time spent in contact with pups (5).

Differences in OT receptor binding in the MPOA between high and low LG females are estrogen dependent (5). Differences in OT receptor binding are eliminated with ovariectomy and reinstated with estrogen replacement (5), and these findings are consistent with earlier reports of the effects of estrogen on OT receptor binding (6, 10). However, whereas ovariectomized high LG females respond to estrogen with an increase in OT receptor binding, there is no such effect in low LG females (5). Studies with knockout mice suggest that estrogen regulation of OT receptor binding in the MPOA requires the α-subtype of the estrogen receptor (ER) (11), and we found significantly increased expression of ERα but not ERβ in the MPOA of lactating high and low LG mothers as well as in their nonlactating, virgin female offspring (12). Considering the importance of ERα activation for the regulation of OT receptor binding in the MPOA and expression of maternal behavior (6–8, 13), we propose that individual differences in maternal behavior are transmitted from the mother to the female offspring through effects of maternal care on ERα expression in the MPOA. The critical question concerns the mechanism whereby variations in mother-pup interactions in early life might stably alter ERα expression in the MPOA.

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Abbreviations: ChIP, Chromatin immunoprecipitation; ER, estrogen receptor; LG, licking/grooming; MPOA, medial preoptic area; NGFI, nerve growth factor-induced protein; OT, oxytocin; ROD, relative OD; SSC, saline sodium citrate; Stat, signal transducer and activator of transcription; TBS-T, Tris-buffered saline with Tween 20.

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ERα is a ligand-activated transcription factor that regulates gene transcription in response to estrogens (14, 15). The cellular response to estrogens is related to the intracellular ER concentrations (15–17). The human ER gene is transcribed from two different promoters, the proximal A and distal B promoters, separated by a 2-kb intron. Sequencing analysis of the 5′ flanking untranslated exon (exon 1b) region of the rat ER gene (18) shows that transcription occurs from a promoter greater than 70% homologous to human ER promoter B, with no evidence for a functional promoter A in the rat ERα gene. Although recent reports suggested the presence of several additional promoter regions for the ERα gene in the human brain (19), ERα mRNA variant transcribed from promoter B the most abundant in neuronal tissue in the rat brain (20). These findings suggest that the exon 1b region contains the elements necessary for transcriptional regulation of constitutive expression of the rat ERα gene in the brain. One pathway through which this expression is regulated involves signal transducer and activator of transcription (Stat)5b response elements present in ERα promoter (21).

In the studies reported here, we used cross-fostering studies to illustrate the association between maternal care and ERα expression in the MPOA. Subsequent analysis of the promoter B sequence in the offspring of high and low LG mothers suggest that the maternal effect on ERα expression in adult offspring is associated with differential methylation of the ERα 1b promoter and altered Stat5 binding.

Materials and Methods

Animals

The animals used were out-bred Long-Evans, hooded rats born in our colony and housed in 46 cm × 18 cm × 30 cm Plexiglas cages that permitted a clear view of all activity within the cage. Food and water were provided *ad libitum*. The colony was maintained on a 12-h light, 12-h dark schedule with lights on at 0800 h. The animals underwent routine cage maintenance beginning on d 7 of life but were otherwise unmanipulated. All procedures were performed according to guidelines developed by the Canadian Council on Animal Care and protocols approved by the McGill University Animal Care Committee. At the time of weaning on d 22 of life, the offspring were housed in same-sex, same-litter groups of two animals per cage. After mating and throughout lactation, adult females were housed singly.

Maternal behavior

Maternal behavior was observed for five, 75-min observation periods daily for the first 6 d postpartum by individuals trained to a high level of interrater reliability (*i.e.* > 0.90) (1–5, 20). Observations occurred at three periods during the light (0900, 1300, 1700 h and two during the dark (0600 and 2100 h) phases of the light-dark cycle. Within each observation period, the behavior of each mother was scored every 3 min (25 observations/period × 5 periods per day = 125 observations/mother per day) for the following behaviors: mother of pups, mother carrying pup, mother LG any pup, mother nursing pups in either an arched-back posture, a blanket posture in which the mother lays over the pups, or a passive posture in which the mother is lying either on her back or side while the pups nurse (see Refs. 22,23 for a detailed description). The frequency of maternal LG across a large number of mothers is normally distributed (22). Hence, high and low LG mothers represent two ends of a continuum, rather than distinct populations. High LG mothers were defined as females whose frequency scores for LG were greater than 1 SD above the cohort mean. Low LG mothers were defined as females whose frequency scores for LG were greater than 1 SD below the cohort mean. Only litters consisting of five or more pups were included in the study.

Cross-fostering

A cohort of 100 nulliparous females were mated and observed during postpartum d 1–6 as previously described (4). With the exception of regular cage maintenance, these females were left undisturbed with their pups until weaning at d 21. From these cohorts 15 high and 15 low LG mothers were selected and remated. The females were assigned to six groups: low (nonfostered), high (nonfostered), low to low, high to high, low to high, and high to low. The high LG females (n = 15) were divided among the high (n = 5), high-to-high (n = 5), and high-to-low groups (n = 5), whereas the low LG females (n = 15) were divided among the low (n = 5), low-to-low (n = 5), and low-to-high (n = 5) groups. Mothers were briefly removed from their cages within the 12-h period after parturition. At that time, all pups were marked on their hind leg with Codman surgical markers (Johnson & Johnson, New Brunswick, NJ). Such marking does not alter rates of maternal LG or arched-back nursing (Mar, A., F. A. Champagne, and M. J. Meaney, unpublished data). From the litter, two male and two female pups were marked on their left hind leg, whereas the remaining pups were marked on their right hind leg. The pups with left hind markings were then transferred to another litter that had given birth within the same 12-h period. In cases in which mothers were assigned to the low-to-low group, the four pups were taken from the low LG mother and given to another low-to-low mother (with reciprocal exchange of pups to the donating female). The same procedure was used within the high-to-high group, with high LG females exchanging four pups with other high LG females in the high-to-high group. In the low to high group, four pups from a low LG mother were fostered to a high LG mother from the high-to-low group which, in turn, had four of her pups fostered to the donor litter. Overall, 24 pups (12 male and 12 female) were cross-fostered for each group. The litters were left undisturbed until weaning with the exception of two 10-min sessions in which the markings on the hind legs were reapplied. These sessions occurred at postnatal d 7 and 14 and coincided with the regular cage changing that occurs once a week in the animal facility. At weaning, cross-fostered pups from each litter were pair housed.

Stat5b Western blot

Whole brains were removed from young (d 6) and adult female adult (d 90) female offspring of high and low LG mothers (n = 5 animals/group) by rapid decapitation less than 1 min after their removal from the home cage. The MPOA was dissected, snap frozen on dry ice and stored at −80 C. The MPOA tissue whole-cell extract was prepared using tissue sonicated on ice (20 sec pulse at 40 C) in TEDGEM (100 ml per 50 g of MPOA tissue) containing NaCl (0.4 M) and protease inhibitors [aprotinin (3.5 mg), 4-(2-aminoethyl) benzenesul-fonylfluouride (0.4 mg/ml), leupeptin (1 mg/ml), and pepstatin 1 mg/ml)]. Aliquots of the supernatant were subsequently taken to determine the levels of protein in the whole MPOA. Samples (40 μg protein) were mixed with an equal volume of 0.125 M Tris base, 20% glycerol, 4% sodium dodecyl sulfate, and 0.005% bromophenol blue and then denatured for 5 min at 100 C and separated on Novex 4–12% Tris-glycine PAGE precast gels (Helixx Technologies, Toronto, Canada) with stained molecular markers (SeeBlue; Invitrogen, Carlsbad, CA) loaded for reference. Aliquots (10 μl) were loaded onto Novex precast gels and subjected to electrophoresis (3 h, 100 V). Proteins were electrophoretically transferred onto nitrocellulose membranes (14 h, 160 mA). The membranes were blocked (1 h, 22 C) with Carnation dried milk (5%) in Tris-buffered saline with Tween 20 (TBS-T) [Tris, NaCl, Tween 20(0.1%) (pH 7.6)], washed briefly in TBS-T, and incubated (14 h, 4 C) with antirat Stat5b monoclonal antibody (catalog no. sc-1656, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in 1:500 with TBS-T.

Membranes were rinsed in 2 × 5-min TBS-T washes and incubated (1 h, 22 C) with horseradish peroxidase-conjugated sheep antimouse IgG antibody (1: 3000; Amersham Pharmacia Biotech, Piscataway, NJ). After 6 × 10-min washes in TBS-T, bands were visualized by enhanced chemiluminescence (Amersham, Aylesbury, UK) and apposed to autoradiography film (Hyperfilm-MP; Amersham) before being developed. A single band was observed at approximately 94 kDa. To verify the accuracy of sample loading, selected blots were incubated (30 min, 70 C) in stripping solution [Tris-HCl (62.5 mM), β-mercaptoethanol (100 mM), sodium dodecyl sulfate (2%)] before being blocked (14 h, 4 C) and reprobed (1 h, 22 C) with an α-tubulin monoclonal antibody (1:5000; Biodesign International, Saco, ME). A single band was observed at approximately 60 kDa, and the intensity of the signal was similar in all

lanes. Relative OD (ROD) readings for the ERα band was determined using a computer-assisted densitometry program (MCID 4.0; Imaging Research, St. Catherine's, Ontario, Canada) from samples run in triplicate on three different blots. For all studies, single blots were derived from samples from one animal and ROD values analyzed using a *t* test.

In situ hybridization

ERα *in situ* hybridization was performed as previously described (24). Whole brains were removed from diestrus adult (90 d old) and 6-d-old female offspring of high and low LG mothers (n = 5 animals/group) by rapid decapitation less than 1 min after their removal from the home cage. The MPOA was dissected, snap frozen on dry ice, and stored at −80 C. Coronal sections (15 μm) corresponding to stereotaxic levels from 0.1 to 0.01 mm from bregma (25) were thaw mounted on poly-L-lysine-coated slides and stored at −80 C until processing. Slides were fixed in paraformaldehyde (4% vol/vol), rinsed twice in PBS, and placed in acetic anhydride (0.25% vol/vol) in triethanolamine (0.1 M)/2× saline sodium citrate (SSC) to reduce nonspecific binding. The slides were then passed through a series of ascending ethanol washes, chloroform (100%), and partially rehydrating ethanol washes and air dried (22 C). ^{35}S-labeled cRNA probes (generously provided by Dr. Serge Rivest, Université de Laval, Québec, Canada) were transcribed using a T3 MAXIscript kit (Ambion, Austin, TX). Diluted probe (100 μl) was applied to each section, and slides were covered with a UV-treated acetate sheet and incubated in sealed plastic container (18 h, 55 C). The acetate sheet was removed and slides were put through two washes in 2× SSC (22 C) followed by a series of single washes: 30 min in RNase (20 μg/ml, 37 C), 15 min in 1× SSC (22 C), 30 min in 0.5× SSC (50 C), 30 min in 0.1× SSC (65 C), and one brief wash in distilled water before being dehydrated. The slides were then exposed to autoradiography film (Hyperfilm-βmax; Amersham) for 4–6 d before being developed. ROD readings were determined (average ROD for n = 3 sections/animal) using a computer-assisted densitometry program (MCID Systems; Imaging Research) and analyzed using a *t* test.

Sodium bisulfite mapping

Sodium bisulfite mapping was performed as previously described (26, 27). Whole brains were removed from adult (90 d old) female offspring of high and low LG mothers (n = 5 animals/group). The MPOA was dissected from whole-brain tissue, and the total DNA from the MPOA was purified and resuspended in 100 μl double-distilled H_2O. Sodium bisulfite solution [NaBis (3.6 M)/hydroquinone (1 mM)] was added to the resuspended DNA and incubated (14 h, 55 C). DNA was eluted (QIAquick, PCR purification kit, QIAGEN, Mississauga, Canada) in Tris buffer [10 mM (pH 8.5)], and NaOH (3 M) was added to a final concentration of 0.3 N NaOH and incubated (15 min, 37 C). NH_4OAc (10 M) was added to a final concentration of 3 M NH_4OA, followed by the addition of tRNA (0.1 mg/ml) and EtOH (2 vol/vol, 95%) were added, and the bisulfited DNA solution was cooled (20 min, −20 C). The precipitated solution was then centrifuged (4 C, 13,200 rpm, 30 min), and the pelleted DNA was lyophilized and resuspended in double-distilled H_2O (50 ng/ ml).

The exon 1b ERα promoter region (GenBank accession no. X98236) of the sodium bisulfite-treated MPOA DNA (50 ng/ml) was subjected to PCR amplification using outside primers (forward: 1874-TAGTATATTTTGATTGTTATTTTAT-1898; reverse: 2377-TCTCAAACCACTCAAACTAC-2358). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 C), 34 cycles of denaturation (1 min, 95 C), annealing (2 min 30 sec, 56 C), and extension (1 min, 72 C), followed by a final extension cycle (5 min, 72 C) terminating at 4 C. The PCR product (504 bp) was used as a template for subsequent PCRs using nested primers (forward: 1899-TTTATTTGTGGTTTATAGATATTT-1922; reverse: 2377-ACAAAAAAAAAAAAATCAAAACAC-2334). The nested PCR product (479 bp) was then subcloned (original TA cloning kit, Invitrogen), transformed, and 10 different clones per plate were miniprepped. Only plasmids containing the exon 1b ERα promoter region were sequenced. Plasmids were screened until 10 positive plasmids were collected per animal (n = 5 animals/group). These 100 positive clones were then sequenced (T7 sequencing kit; USB, Amersham Pharmacia Biotech) starting from procedure C in the manufacturer's protocol and included in the subsequent analysis. The sequencing reactions were resolved on a denaturing (6%) polyacrylamide gel and visualized by autoradiography. The methylation status of individual CpG sites across the exon 1b ERα promoter was averaged per animal per group, and the percentage of methylated cytosine residues (mean ± SEM) were statistically compared between groups by ANOVA. Repeated-measures ANOVA was used to analyze group by region effects.

Stat5b chromatin immunoprecipitation (ChIP) assay

In the preparation of fixed tissue, adult (90 d old) female offspring of high and low LG mothers (n = 4 animals/group) were deeply anesthetized with sodium pentobarbital (60 mg/kg, ip) and then transcardially perfused with heparinized saline flush (30–60 ml), followed by paraformaldehyde (4%) in phosphate buffer saline (pH 7.4) for 15 min. After perfusion, all brains were removed and postfixed in the same fixation solution (14 h, 4 C) and then transferred to phosphate buffer sucrose (20%) for 48 h. ChIP assays (28) were performed after the ChIP assay kit protocol (catalog no. 06-599; Upstate Biotechnology, Lake Placid, NY). The MPOA was dissected from each rat brain and chromatin was immunoprecipitated using 10 μg of either antirat Stat5b monoclonal antibody (catalog no. sc-1656; Santa Cruz Biotechnology) or normal mouse IgG nonimmune antibody (catalog no. sc-2025; Santa Cruz Biotechnology). One tenth of the lysate was kept to quantify the amount of DNA present in different samples before immunoprecipitation (Input). The rat MPOA exon 1b ERα promoter region (GenBank accession no. X98236) of the uncross-linked DNA was subjected to PCR amplification (forward primer: 1836-GAAGAAACTCCCCTCAGCAT-1855; reverse primer: 2346-GAAATCAAAACA CCGATCCT-2327). PCRs were done with the FailSafe PCR system protocol using FailSafe PCR 2 × PreMix A (Epicenter; InterScience, Markham, Canada). The thermocycler protocol involved an initial denaturation cycle (5 min, 95 C), 34 cycles of denaturation (1 min, 95 C), annealing (1 min, 60 C), and extension (1 min, 72 C), followed by a final extension cycle (10 min, 72 C) terminating at 4 C. PCRs on DNA purified from nonimmunoprecipitated samples and immunoprecipitated samples were repeated exhaustively using varying amounts of template to ensure that results were within the linear range of the PCR.

Products were separated on an agarose gel (2%) to visualize bands corresponding to the exon 1b ERα promoter (493 bp) DNA fragment. Nucleic acids were transferred by Southern blot (14 h, 22 C) to positively charged nylon transfer membrane (Hybond-N^+, Amersham). An oligonucleotide (20 bp) specific for the exon 1b ERα promoter sequence (GenBank accession no. X98236; forward: 1942-AGAAAGCACTGGACATTTCT-1961) was radiolabeled [1 μl T_4 polynucleotide kinase, Promega, Madison, WI] with 5 μl [γ^{32}P]ATP (Amersham) (2 h, 37 C) and then hybridized to the membranes that were then subjected to autoradiography. ROD readings were determined using a computer-assisted densitometry program (MCID Systems; Imaging Research). To calculate the final signal for each sample, the ROD value of the band within the antibody lane (A) was divided by the ROD value of the band within the input lane (I). Group comparisons were made statistically using a *t* test.

Results

Developmental studies

The adult virgin female offspring of high and low LG mothers differ in ERα expression in the MPOA (7). We examined whether such differences emerged in early postnatal life. The results of *in situ* hybridization studies reveal that ERα expression in the MPOA of the postnatal d 6 offspring of high LG mothers is significantly [$t_{(8)} = 4.56$, $P < 0.01$] increased by comparison with that of the offspring of low LG mothers (Fig. 1).

Cross-fostering studies

The adult offspring of high LG mothers show increased ERα expression in the MPOA; however, such findings are correlational and do not imply a direct relation between maternal behavior and ERα expression. We addressed this

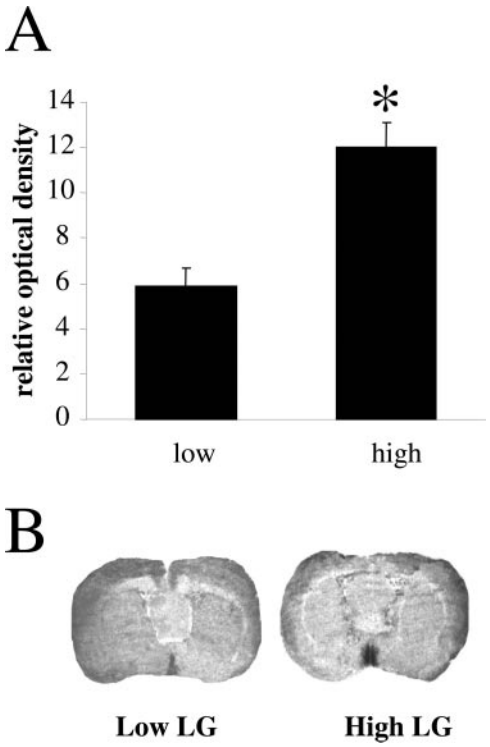

FIG. 1. A, ERα mRNA (ROD units ± SEM) in the MPOA of d 6 female offspring of low and high LG mothers. At d 6, ERα expression differences between groups is apparent, with elevated levels in the offspring of high LG, compared with low, LG mothers ($P < 0.01$). B, Representative photomicrographs indicating ERα expression in the MPOA of d 6 female high and low LG offspring.

issue using a cross-fostering paradigm in which the biological offspring of high and low LG mothers were reciprocally cross-fostered within 12 h of birth. ERα expression in the MPOA of the adult offspring (Fig. 2) was examined using *in situ* hybridization and revealed a highly significant effect of rearing mother [$F_{(1, 26)} = 31.73$, $P < 0.001$] that provides evidence for a direct relation between maternal care and ERα expression in the MPOA in the adult female offspring. Thus, ERα expression in the MPOA of adult females born to low LG mothers, but reared by high LG dams, was indistinguishable from that of the normal offspring of high LG mothers.

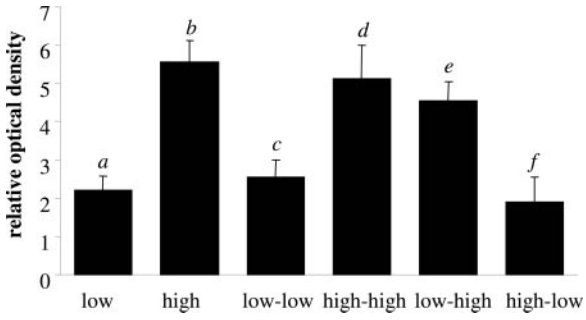

FIG. 2. ERα mRNA (ROD units ± SEM) in the MPOA of adult females who had been cross-fostered to high or low LG mothers postpartum. Females cross-fostered from a high or low LG mother to a low LG mother, had equivalent levels of ERα mRNA and had less ER expression on the MPOA than females cross-fostered from a high or low LG mother to a high LG mother. a *vs.* b, $P < 0.01$; c *vs.* d, $P < 0.05$; e *vs.* f, $P < 0.05$; b *vs.* d *vs.* e, n.s.; a *vs.* c *vs.* f, n.s.

Similarly, ERα expression in the MPOA of adult females born to high LG mothers, but reared by low LG dams, was indistinguishable from that of the normal offspring of low LG mothers. Note that the cross-fostering procedure alone had no effect on ERα expression (Fig. 2). Thus, ERα mRNA levels in the adult offspring born to high LG and fostered to another high LG mother did not differ from that of nonfostered offspring of high LG mothers. The same was true for the offspring of low LG mothers.

Methylation of the ER1b promoter

These cross-fostering data suggest an enduring, epigenetic effect of maternal care on ERα expression in the female rat and beg the question of mechanism. We showed that expression of the glucocorticoid receptor in the hippocampus is also epigenetically programmed by maternal care (29, 30). Increased maternal LG is associated with site-specific demethylation of the recognition sequence for the transcription factor nerve growth factor-induced protein (NGFI)-A in a glucocorticoid receptor promoter. Cytosine methylation is a stable mark on DNA associated with decreased gene expression (31) and, we propose, a possible mechanism for long-term effects of maternal care on gene expression. We therefore examined whether a similar mechanism is involved in maternal programing of ERα expression in the MPOA of female offspring. We delineated the methylation status of individual CpG sites across the ERα1b promoter using sodium bisulfite mapping (26, 27) with MPOA samples obtained from the adult offspring of high and low LG mothers. The results reveal highly significant differences in the methylation status of individual CpG sites across the entire ERα 1b promoter (Fig. 3B). Overall, significantly elevated levels of methylation were detected in the promotor of the offspring of low LG dams, compared with offspring of high LG dams [$t_{(8)} = 4.06$, $P < 0.01$; Fig. 3A]. Repeated-measures ANOVA indicated a main effect of maternal care [$F_{(1, 8)} = 16.53$, $P < 0.01$] and a main effect of region [$F_{(13, 104)} = 41.72$, $P < 0.001$] on methylation of the ERα promotor in the MPOA. *Post hoc* analysis indicated that significant ($P < 0.05$) group differences were present at sites 1, 3, 4, 7, 9, 10, and 11 (Fig. 3B), and in each case the frequency of cytosine methylation was greater in the offspring of low LG mothers.

Stat5b binding to the ER1b promoter

Among the CpG sites differentially methylated in the offspring of high and low LG mothers was one (site 3) contained within a consensus Stat5b binding site. ERα expression is regulated through activation of the Janus kinase-Stat5b pathway (21). One mechanism by which DNA methylation suppresses gene expression is by inhibiting transcription factor binding to its methylated binding site. To examine the possible functional significance of the differences in the methylation of the CpG site within the Stat5b binding site, we determined whether the differences in promoter methylation were associated with differences in the occupancy of the ERα promoter by Stat5b. We performed a ChIP assay with MPOA tissue from paraformaldehyde-perfused animals from which cross-linked protein-DNA complexes were immunoprecipitated with a Stat5b antibody. Complexes were then unlinked

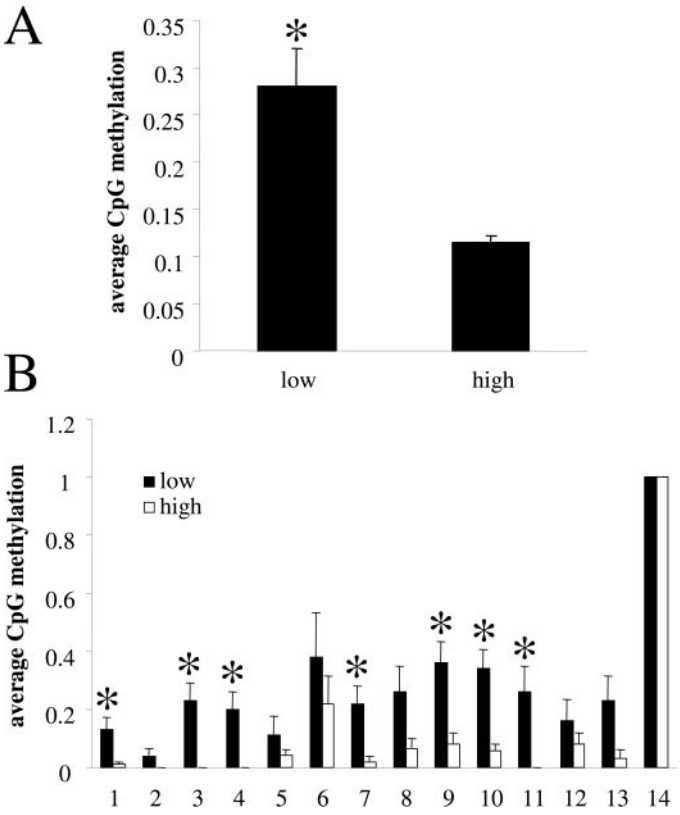

FIG. 3. A, Average cytosine methylation (± SEM) of the 1b ERα promoter region in the adult female offspring of high and low LG mothers. Overall, methylation was elevated in low, compared with high, LG offspring ($P < 0.01$). B, Average cytosine methylation of the 14 CpG sites within the 1b ERα promoter region in the adult female offspring of high and low LG mothers. Significant group differences were found at regions 1, 3, 4, 7, 9, 10, and 11 ($P < 0.05$), with female offspring of low LG mothers having elevated levels of methylation at each of the seven sites.

and the precipitated genomic DNA was subjected to PCR amplification with primers specific for the ERα promoter 1b sequence. The results indicated significantly [$t_{(8)} = 3.2$, $P < 0.05$] greater binding of Stat5b to the MPOA ERα1b promoter in the adult offspring of high, compared with low, LG mothers (Fig. 4). Thus, maternal care is associated with ERα expression, DNA methylation of the 1b promoter and alterations in Stat5b binding.

Maternal effects on Stat5b immunoreactivity

ERα expression is regulated by a number of hormonal signals, including prolactin and IGF-1, both of which were previously shown to activate Stat5b expression. Indeed, the effect of prolactin on ERα expression in the rat corpus luteum is mediated by Stat5b binding (21). Because ERα expression in the MPOA of the offspring high and low LG mothers differs as early as d 6 of postnatal life, we examined Stat5b levels in neonates. Western blots with MPOA from d 6 offspring revealed a highly significant [$t_{(6)} = 4.03$, $P < 0.01$] increase in Stat5b in the offspring of high, compared with low, LG mothers (Fig. 5). In contrast, there was no effect of maternal care on Stat5b immunoreactivity in the MPOA of adult offspring [$t_{(6)} = 1.37$, n.s.].

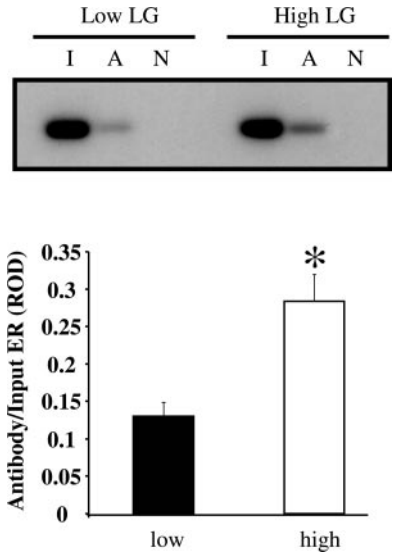

FIG. 4. ChIP of MPOA samples from four high and four low adult female offspring of high and low LG mothers. The 1b ERα promoter was incubated with Stat5b antibody. Binding of Stat5b to the 1b ERα promoter was decreased in the samples obtained from low LG, compared with high LG, female offspring ($P < 0.05$). I, Nonimmunoprecipitated input; A, primary antibody immunoprecipitated; N, nonimmune IgG antibody immunoprecipitated MPOA extracts.

Discussion

The results of the cross-fostering study reveal an association between maternal care and ERα expression in the MPOA. Thus, ERα expression in the MPOA of the biological female offspring of low LG mothers fostered to high LG dams was comparable with that of the normal offspring of high LG mothers. Likewise, ERα expression in the MPOA of the biological offspring of high LG mothers reared by low LG dams was indistinguishable from that of the normal offspring of low LG mothers. The results of the developmental study

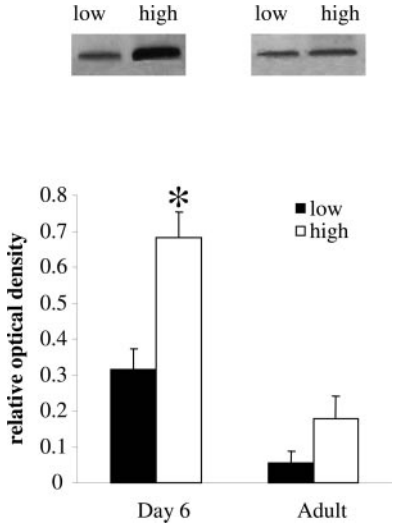

FIG. 5. Stat5b protein levels (ROD units ± SEM) in MPOA samples derived from d 6 and adult female offspring of high and low LG mothers. Stat5b immunoreactivity was elevated in d 6 offspring of high LG mothers, compared with low LG offspring ($P < 0.01$); however, group differences were not significant in adult females.

show that the maternal effect on ERα expression in the MPOA is apparent by postnatal d 6. Differences in pup LG between high and low mothers are apparent between d 1 and 8 postpartum but not thereafter (6, 7, 9). Taken together with the results of the cross-fostering study, these findings suggest that individual differences in ERα expression in the MPOA emerge over the first week of life in association with variations in maternal behavior.

Individual differences in maternal behavior are transmitted across generations. The adult female offspring of high LG mothers display increased pup LG, compared with the adult female offspring of low LG mothers. Cross-fostering reverses this association, suggesting an epigenetic mechanism for the of inheritance individual differences in maternal behavior (4, 5). The maternal effect on ERα expression in the MPOA may serve as a mechanism for differences in maternal behavior of female offspring. The activation of ERα in the MPOA enhances OT receptor binding (6, 8, 10, 11), and this effect is essential for the onset of maternal behavior in the rat (6). Infusions of either estrogen (32) or OT (33) directly into the MPOA facilitate the expression of maternal care. OT receptor binding in the MPOA of lactating high LG mothers is significantly greater than that of low LG dams and central infusion of an OT receptor antagonist eliminates the group difference in pup LG (5). The difference in OT receptor binding in the MPOA is estrogen dependent (6, 8, 10, 32). In the absence of estrogen or in diestrus virgin females, there are no differences in OT receptor levels (5, 9). In ovariectomized animals, estrogen replacement at levels that mimic those of late pregnancy (34) induces a dose-related increase in OT receptor levels in the MPOA in the offspring of high LG mothers but is without effect on the offspring of the low LG dams (5).

The same group differences in estrogen sensitivity are apparent in studies of estrogen-induced cFOS expression in the MPOA (12). Variations in estrogen sensitivity are likely mediated by tissue-specific differences in ERα expression in the MPOA. Lactating high LG mothers show increased ERα expression in the MPOA, compared with lactating low LG dams; such differences are apparent at the level of both mRNA and protein, with no differences in the expression of ERβ (12). Finally, the differences in ERα expression in the MPOA are apparent in the virgin female offspring of high and low LG mothers and are completely reversed with cross-fostering (Fig. 2). Our findings suggest a maternal effect on ERα expression in the MPOA that might mediate the transmission of individual differences in maternal behavior from mother to female offspring. The role of neonatal estradiol levels in mediating the differences in expression of ERα in the MPOA observed in offspring of high and low LG dams has not been thoroughly evaluated, although this may provide an additional mechanism of maternal effects on gene expression.

Whereas differences in maternal behavior between high and low LG mothers are apparent only between d 1 and 8 postpartum, differential ERα expression in the MPOA is observed in adult animals. These findings suggest a maternal programming effect on ERα and the critical question concerns the mechanism for such a programming effect during early postnatal life. Maternal effects on steroid receptor expression in the rat are not unique to ERα expression in the MPOA. The male offspring of high LG mothers show increased glucocorticoid receptor expression in the hippocampus, an effect that is associated with differences in hypothalamic-pituitary-adrenal response to stress (1, 29). The increased glucocorticoid receptor expression in the hippocampus is associated with increased activity of a brain-specific exon 1_7 promoter, which contains an NGFI-A response element (29, 30, 35). Binding of NGFI-A to the exon 1_7 promoter enhances gene expression. Neonates reared by high, compared with low, LG mothers show increased hippocampal NGFI-A expression and increased NGFI-A binding to the exon 1_7 sequence. The increased NGFI-A binding at d 6 associated with maternal LG is related to cytosine demethylation (36) of the NGFI-A response element that, in turn, influences the affinity of the site for NGFI-A in the adult offspring. Increased cytosine methylation, found in offspring of low LG mothers, is associated with decreased histone acetylation and NGFI-A binding as well as reduced glucocorticoid receptor expression (29, 30). Importantly, infusion of the adult offspring of low LG mothers with trichostatin A enhances histone 3 acetylation and NGFI-A binding to the glucocorticoid receptor promoter, increasing glucocorticoid receptor expression and eliminating the group differences in hypothalamic-pituitary-adrenal responses to stress (29). These findings suggest a relationship between the maternal effects on cytosine methylation and gene expression.

Activation of the Janus kinase-Stat pathway increases ERα expression (21), although effects in brain structures have not been examined. Cytosine methylation is associated with decreased transcription factor binding to certain DNA consensus sequences (31). The differences in cytosine methylation of the Stat5b consensus sequence suggest differences in Stat5b binding in adult offspring as a function of maternal care, and this is exactly what was found (see Fig. 4). Among adult animals, there is increased Stat5b binding to the ERα promoter 1b in the MPOA of offspring of high, compared with low, LG mothers despite the absence of any difference in Stat5b levels in the adult offspring of high and low LG mothers. The differences in Stat5b binding to the ERα promoter 1b presumably reflect the differences in the methylation status of the consensus sequence, although this must be directly confirmed as is the functional contribution of promoter B-derived ERα mRNA because they are not the only ERα transcripts expressed in the rat brain (37). We propose that despite comparable levels of Stat5b expression in MPOA in adulthood, the difference in the methylation status of the Stat5 consensus sequence associated with variations in maternal behavior influence Stat5 binding such that occupancy of the Stat5 site on the ERα 1b promoter is increased in the offspring of the high LG mothers, leading to increased ERα expression and thus differences in OT receptor induction in the MPOA and maternal behavior. This process could explain, in part at least, the epigenetic transmission of individual differences in maternal behavior from the mother to her female offspring.

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