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Natural Variations in Maternal Care Are Associated with Estrogen Receptor α Expression and Estrogen Sensitivity in the Medial Preoptic Area

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Lactating rats exhibit stable individual differences in pup licking/grooming (LG) over the first week postpartum. Such naturally occurring variations in maternal behavior are associated with differences in estrogen-inducible oxytocin receptors in the medial preoptic area (MPOA) of the hypothalamus. We compared levels of ERα and ERβ mRNA in the MPOA of lactating High or Low LG mothers as well as in their nonlactating, female offspring, which inherit the maternal phenotype of their mothers. Among lactating females, High LG females exhibited significantly elevated levels of ERα mRNA compared with Low LG females. Likewise, the adult, virgin female offspring of High LG mothers showed higher levels of ERα mRNA in the MPOA compared with those of Low LG mothers. There were no group differences in levels of ERβ mRNA. Differences in ERα protein expression in the MPOA were confirmed using Western blot analysis. To further characterize the effects of estrogen in the MPOA, c*Fos* immunoreactivity was compared in ovariectomized, adult offspring of High and Low LG dams treated with estradiol or oil. Increased cFos activity in the anterior ventral nucleus of the MPOA was observed in estradiol-treated High LG, but not Low LG females. These findings suggest that natural variations in maternal care are associated with differences in ERα expression in the MPOA and that such differences are transmitted from the mother to her female offspring. (*Endocrinology* 144: 4720–4724, 2003)

NATURAL 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) exhibit more modest hypothalamic-pituitary-adrenal responses to stress and enhanced cognitive ability (1–4). These variations in maternal care appear to be transmitted across generations, such that the offspring of High LG mothers are themselves high in LG, whereas the offspring of Low LG mothers are Low LG mothers (4).

In the rat, central oxytocin (OT) receptor levels are functionally linked to behavioral differences in maternal care (5–7), and OT receptor binding in the medial preoptic area (MPOA) of the hypothalamus is increased in lactating females that exhibit High LG compared with Low LG (5, 6). 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 lactating High and Low LG females and their nonlactating female offspring are estrogen dependent. 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 (8, 9). However, although ovariectomized virgin offspring of High LG females respond to estrogen with an increase in OT receptor binding, there is no effect in the offspring of Low LG females. Studies with knockout mice suggest that estrogen regulation of OT receptor binding in the MPOA requires the α-subtype of the estrogen receptor (ER) (10). On the basis of these findings, we examined the expression of ERα and ERβ in the MPOA of lactating High and Low LG mothers and their nonlactating, virgin female offspring. The results suggest group differences in ERα expression in the MPOA that are associated with differences in estrogen sensitivity (examined using cFos immunohistochemistry) and are transmitted to the female offspring.

Materials and Methods

Subjects

The animals used were adult (d 70–100), Long-Evans hooded rats (derived from animals obtained from Charles River Breeding Laboratories, St. Constant, Quebec, Canada), born in our colony and housed in $46 \times 18 \times 30$-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 12 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 weaning on d 22 of life, the offspring were housed in same-sex, same-litter groups of two animals per cage until the time of testing. After mating and throughout lactation, or after surgical interventions, adult females were singly housed. All experiments were performed by individuals who were blind to the developmental history of the animals.

Abbreviations: c*Fos*-ir, c*Fos* immunoreactive; ER, estrogen receptor; LG, licking/grooming; MPOA, medial preoptic area; OT, oxytocin; PKA, protein kinase A; PKC, protein kinase C; RT, room temperature; SSC, saline sodium citrate; TBS, Tris-buffered saline; vBNST, ventral region of the bed nucleus of stria terminals.

Maternal behavior

Maternal behavior was examined as previously described (11, 12). The behavior of each dam was observed for six 75-min daily observation periods for the first 6 d postpartum. Observers were trained to a high level of interrater reliability (*i.e.* 0.90). Observations occurred at regular times each day, with four periods during the light (0900, 1200, 1500, and 1800 h) and two periods during the dark (0600 and 2100 h) phases of the light/dark cycle. Within each 75-min observation period, the behavior of each mother was scored every 3 min (25 observations/period × six periods/d = 150 observations/mother per day) for the following behaviors (11, 12): mother off pups, mother carrying pup, mother licking and grooming any pup, or mother nursing pups in 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.

The frequency of maternal LG and arched-back nursing across a large number of mothers is normally distributed (11). Hence, High and Low LG mothers represent two ends of a continuum, rather than distinct populations. To define these populations for the current studies, we observed the maternal behavior in cohorts of mothers, ranging from 30–40 dams with their pups, and devised the group mean and SD for each behavior over the first 6 d of life as previously described (11). High LG mothers were defined as females whose frequency scores for LG were greater than 1 SD above the mean. Low LG mothers were defined as females whose frequency scores for LG were greater than 1 SD below the mean.

Ovariectomy and estradiol replacement

Animals were anesthetized and bilaterally ovariectomized. At the time of surgery, the animals were implanted SC with a single SILASTIC brand capsule [Dow Corning (Midland, MI); 1.98 mm inner diameter, 3.17 mm outer diameter; 10 mm in length per 100 g body weight] containing 0 or 10 mg estradiol benzoate (β-estradiol 3 benzoate; Sigma, St. Louis, MO) dissolved in peanut oil vehicle. We (5, 13) previously found that this treatment produces plasma estradiol levels (~70 pg/ml) that mimic those of late pregnancy (14, 15). Four days after ovariectomy, animals were perfused through the heart with 150 ml of 0.9% saline followed by 150 ml of 4% paraformaldehyde (Sigma) dissolved in 0.1 M sodium PBS (pH 7.4). The 4-d treatment period was selected on the basis of previous studies examining the effects of estrogen on OT receptor binding and cFos immunoreactivity (5, 15, 16). Brains were removed, postfixed overnight in 4% paraformaldehyde, and submerged in 30% sucrose in 0.1 M PBS for 3 d at −4 C before sectioning.

cFos immunohistochemistry

Perfused whole brains were sliced coronally into 40-μm sections and stored in cryoprotectant (pH 7.4) until processing. Free-floating sections were transferred into wells and washed three times in 0.9% Tris-buffered saline (TBS, pH 7.6) for 5 min each rinse. After the final rinse, sections were incubated for 30 min in 30% hydrogen peroxide in TBS at 4 C. After incubation, sections were rinsed in three 5-min washes in TBS, incubated for 90 min in 3% normal goat serum in 0.3% Triton TBS, and then incubated for 48 h at 4 C in 0.3% Triton TBS with 3% normal goat serum containing rabbit polyclonal anti-cFos antibody that recognizes residues 4–17 of the human Fos protein (Ab-5, PC-38, Oncogene Research Products, San Diego, CA). Sections were rinsed with 3 × 5-min washes in TBS and incubated for 1 h in 0.3% Triton TBS with 3% normal goat serum containing secondary antibody (ABC Kit, 1:200, antirabbit made in goat, Vector Laboratories, Burlingame, CA). After three 5-min washes in TBS, sections were incubated for 2 h in 0.9% TBS containing tertiary antibody. Finally, sections were removed from tertiary solution and rinsed with three 5-min washes in TBS, and visualized with DAB solution (Vector Laboratories). Sections were rinsed in two 5-min washes with water and stored in TBS until mounted. All sections were mounted on gelatin-coated slides, dehydrated, and coverslipped. cFos counts were determined from three sections per animal using a computer-assisted densitometry program (MCID Systems; Imaging Research, St. Catherines, Ontario, Canada).

Western blot

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, Ontario, Canada) with stained molecular markers (SeeBlue; Invitrogen, Carlsbad, CA) loaded for reference. Then, 10-μl aliquots were loaded onto Novex pre-cast gels and subjected to electrophoresis for 3 h at 100V. Proteins were then electrophoretically transferred onto nitrocellulose membranes overnight at 160 mA. Membranes were blocked for 1 h on a rotator at room temperature with 5% Carnation dried milk (Nestlé, Vevey, Switzerland) in TBS-T [Tris, NaCl, 0.1% Tween-20 (pH 7.6)], washed in TBS-T for 5 min and incubated at 4 C with antirabbit ERα polyclonal antibody (SC-542, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in TBS-T. Membranes were rinsed in 2 × 5-min TBS-T washes and incubated for 2 h at room temperature with antirabbit horseradish peroxidase in TBS-T. After six 10-min washes in TBS-T, bands were visualized by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden) and exposed to Hyperfilm (Amersham Biosciences) before being developed. To check sample loadings, selected blots were stripped and reprobed with β-actin monoclonal antibody. OD readings for ERα band (~66 kDa) were determined using an image-analysis system (MCID-4, Imaging Research).

In situ hybridization

ERα and ERβ *in situ* hybridization was performed as described by Laflamme *et al.* (17) using ^{35}S-labeled cRNA probes generously provided by Dr. Serge Rivest (Centre hospitalier de l'Universite Laval, Québec, Canada). Brains were sliced coronally, 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 [room temperature (RT)]. ^{35}S-labeled cRNA probes 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. Slides were placed in sealed plastic boxes and were subsequently incubated for 18 h at 55 C. Consequently, the acetate sheet was removed, and slides were put through two washes in 2× SSC (RT) followed by a series of single washes: 30 min in Rnase (20 μg/ml, 37 C), 15 min in 1× SSC (RT), 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-β max, Amersham Biosciences) for 4–6 d, before being developed. Relative OD readings were determined from six sections per animal using a computer-assisted densitometry program (MCID Systems; Imaging Research).

Results

ERα and ERβ in situ hybridization

Levels of ERα and ERβ mRNA were examined in the MPOA of lactating (d 6 postpartum) High and Low LG dams (n = 5/group; Fig. 1A). Two-way ANOVA indicated a main effect of group (High *vs.* Low, $P < 0.05$), a main effect of receptor subtype ($P < 0.01$), and a significant group by receptor subtype interaction ($P < 0.05$). *Post hoc* analyses indicated significantly elevated levels of ERα in the MPOA of High LG compared with Low LG dams ($P < 0.05$). No differences were observed in ERβ levels between High and Low LG dams, and no group differences in ERα or ERβ mRNA levels were found in the ventral region of the bed nucleus of the stria terminals (vBNST; Fig. 1A). Levels of ERα were then compared in the MPOA of virgin, adult female (diestrus) offspring of High and Low LG dams (n = 5/group). Analysis revealed a significant difference in ERα mRNA levels be-

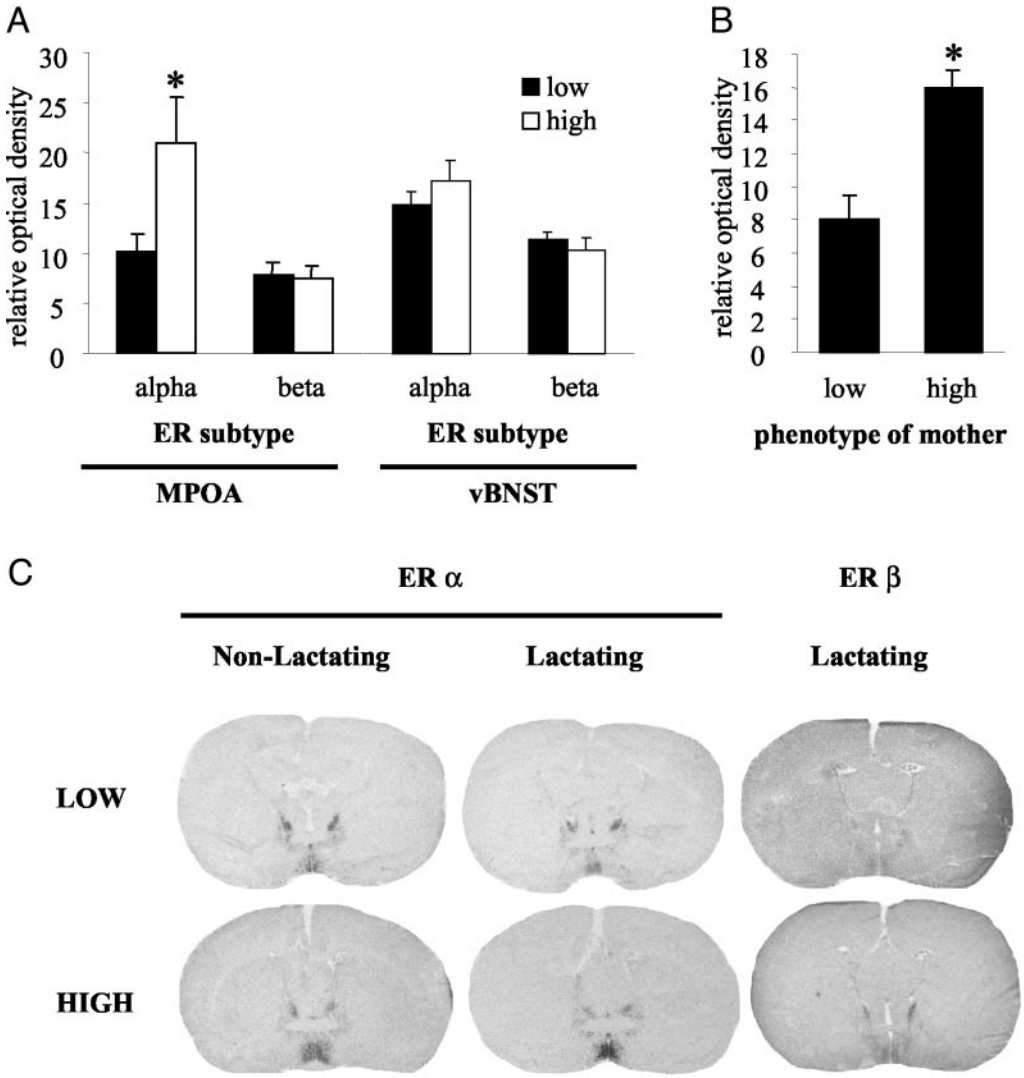


FIG. 1. A, Mean ± SEM. ERα and ERβ mRNA levels in the MPOA and vBNST of High and Low d 6 postpartum lactating females. In the MPOA, two-way ANOVA indicated a main effect of group [F = 5.78, df (1,17); $P < 0.05$], a main effect of probe [F = 12.74, df (1,17); $P < 0.01$], and a group X probe interaction (F = 6.4; $P < 0.05$). No significant differences were found in the vBNST. B, Mean ± SEM. ERα mRNA levels in the MPOA of adult nonlactating (diestrus) female offspring of High and Low LG mothers. Analysis indicated significantly elevated levels of ERα mRNA in the offspring of High LG mothers [t = 4.43, df (9); $P < 0.001$]. C, Representative photomicrographs of ERα and ERβ mRNA levels in High and Low LG lactating and nonlactating females.

tween nonlactating, virgin female offspring of High and Low LG dams ($P < 0.05$; Fig. 1B).

Western blot analysis

Protein levels of nuclear ERα were compared in MPOA extracts from the adult, virgin female (diestrus) offspring of High and Low LG dams (n = 5/group). Significantly elevated levels of ERα-immunoreactivity were found in the female offspring of High LG dams ($P < 0.01$; Fig. 2).

cFos immunohistochemistry

cFos expression was compared in the preoptic area of oil-treated and estradiol-treated ovariectomized adult virgin female offspring of High and Low LG dams (n = 5/group). At the level of the MPOA, cFos immunoreactive (c*Fos*-ir) cells were only detected in the preoptic area and lateral septum. Two-way ANOVA indicated a significant main effect of treatment ($P < 0.05$) and a group X treatment interaction ($P < 0.05$) in the anterior ventral nucleus of the preoptic area, but no significant differences in cFos-ir in more medial regions of the preoptic area (although group differences approach significance in this region, $P < 0.10$) or the lateral septum. *Post hoc* analysis indicated that the virgin, female offspring of Low LG dams treated with estradiol did not show elevations in c*Fos*-ir compared with oil-treated controls. Female offspring of High LG dams treated with estradiol show marked increases in the number of cFos-ir positive cells compared with oil-treated controls ($P < 0.05$; Fig. 3, A and B).

Discussion

Naturally occurring variations in maternal behavior are associated with differences in OT receptor levels in the

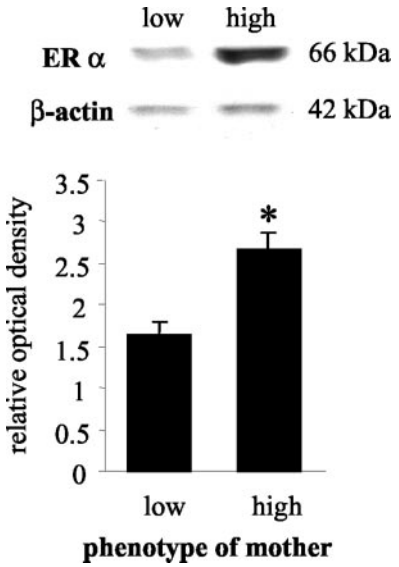
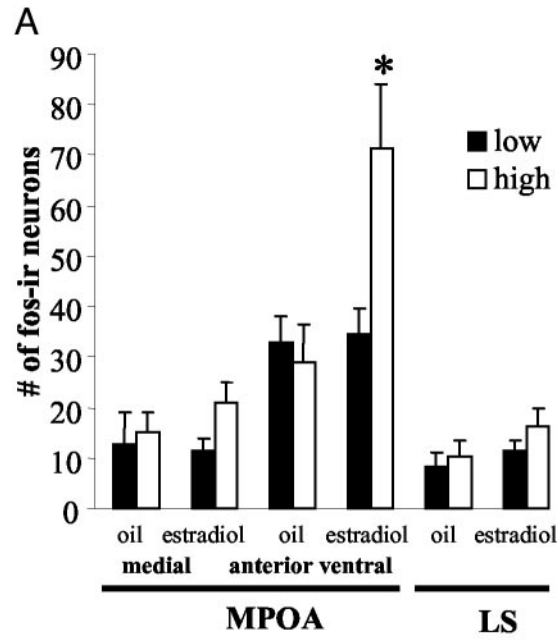


FIG. 2. Mean ± SEM. ERα protein levels in the MPOA of adult female (diestrus) offspring of High and Low LG mothers. Analysis indicated a significant elevation in the levels of ERα protein in the offspring of High LG mothers [t = 3.74, df (9); $P < 0.01$].



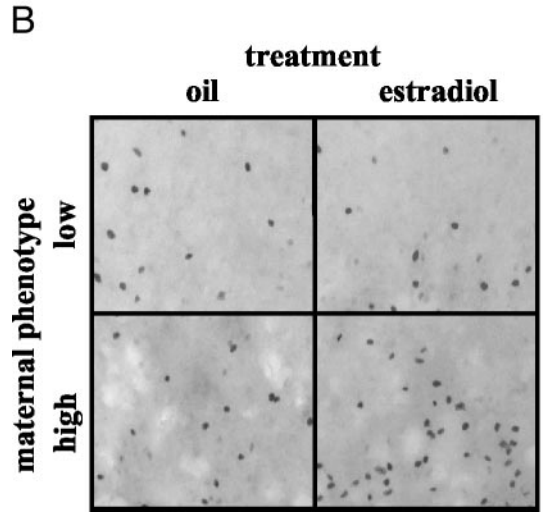


FIG. 3. A, Mean ± SEM. cFos-ir counts in the MPOA and lateral septum of estradiol or oil-treated ovariectomized female offspring of High and Low LG mothers. Two-way ANOVA indicated a main effect of treatment [F = 7.1, df (1,21); $P < 0.05$] and a significant treatment by group interaction (F = 6.1; $P < 0.05$) in the anterior ventral nucleus of the MPOA. *, Female offspring of High LG dams treated with estradiol show marked increases in the number of c*Fos*-ir positive cells compared with oil-treated controls ($P < 0.05$). No significant group or treatment effects were found in the lateral septum or the medial nucleus of the MPOA ($P < 0.10$). B, Representative photomicrographs of cFos-ir cells in the MPOA of oil- and estradiol-treated ovariectomized female offspring of High and Low LG mothers.

MPOA (5, 6). Estrogen regulates OT receptor binding in several brain regions (8, 9), and we have previously reported that differences in OT receptor levels in the MPOA of lactating High and Low LG females and their virgin female offspring are dependent on the presence of circulating levels of estrogen (5, 6). Ovariectomy eliminates the group differences in OT receptor levels in the MPOA, and this effect is reversed with estrogen replacement (5). Interestingly, in the MPOA, estrogen had no effect on OT receptor binding in offspring of Low LG females. These findings are consistent with the results of the present study and suggest that group differences in ERα expression in the MPOA may serve as a mechanism for the differences in OT receptor binding and maternal behavior between lactating High and Low LG females. However, it should be noted that differences in ERα expression also occur in regions of the MPOA where there are no group differences in OT receptor levels, suggesting the possibility that ERα may also influence maternal behavior through mechanisms that are not directly related to OT.

In the rat, estrogen increases maternal responsivity to pups in both nonlactating and pregnancy-terminated females. Estrogen is also required for OT-induced increases in maternal responsiveness (18). Estrogen increases OT receptor mRNA levels in the hypothalamus (8, 9). Several estrogen response element half-sites are present in the promotor region of the OT receptor (19), and estrogen regulation of OT receptor expression appears to involve ERα (10). In general, ERα is more highly expressed in the MPOA relative to ERβ (17, 20) and may be more closely associated with dynamic variations in gene expression (21).

Estrogen also appears to regulate OT receptor binding levels through the activation of protein kinase A (PKA) and protein kinase C (PKC) pathways. Bale *et al.* (22) illustrated PKA and PKC involvement in OT receptor binding and have shown that the regional specificity of estradiol-induced upregulation of OT receptor binding is dependent on the tissue-specific effects of PKC and PKA. Thus, estrogen can regulate OT receptor levels either directly through genomic effects or indirectly through intracellular signaling pathways (23).

Differences in ERα expression in the MPOA were also apparent in the female offspring of High and Low LG mothers. As adults, the lactating female offspring of High LG mothers exhibit significantly higher levels of pup LG than do

the offspring of Low LG mothers (4). These differences are reversed with cross-fostering shortly after birth, suggesting a nongenomic mechanism of transmission of individual differences in maternal behavior. Likewise, when tested as virgin adults, the female offspring of High LG mothers show significantly shorter latencies in the pup sensitization test (14) compared with the offspring of Low LG mothers (5). Recent findings suggest that tactile stimulation associated with LG might serve as the mechanism by which maternal behavior directly influences ERα expression and maternal care in the female offspring. McCarthy *et al.* (24) demonstrated that tactile stimulation (with a paintbrush) of postnatal d 3 pups resulted in a rapid induction of Fos immunoreactivity in the ventral MPOA. In adult animals that were reared artificially (25), we found that tactile stimulation applied over the first week of life increased both ERα mRNA and OT receptor binding in the MPOA, as well as maternal LG (Champagne, F. A., S. Rees, M. J. Meaney, and A. Fleming, unpublished data). These findings suggest that individual differences in ERα mRNA and OT receptor binding in the MPOA as well as those in maternal LG are transmitted to female offspring through the effects of tactile stimulation derived from LG.

These group differences in ERα expression appear to be functionally related to differences in estrogen sensitivity. Estrogen has been previously reported to increase cFos expression in the MPOA (16). In the present study, we found that estrogen replacement in ovariectomized virgin female offspring of High, but not Low, LG mothers produced a significant increase in cFos immunoreactivity. As in the previous studies of Sheehan and Numan (26), we found no effect of estrogen on cFos expression in the lateral septum. The regional specificity of estrogen effects on neuronal activity has been linked to the differential expression of ERα. Double-labeling studies have indicated that in estrogen-treated females, cFos-positive cells are colocalized with ERα and that this colocalization is found primarily in the ventral region of the MPOA (27). Neuronal activation in the MPOA can also be achieved when lactating female rats are exposed to pups, suggesting that maternal stimuli can trigger the activation of ERα-containing cells (28, 29). This stimuli-mediated ERα activation may explain the maintenance of high levels of OT gene expression during the first week postpartum, despite the significant decline in estrogen levels that characterizes this period (30). Although this remains a matter of speculation, these findings provide a cellular mechanism for the differences in estrogen sensitivity in the MPOA between the offspring of High and Low LG females.

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