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Coexpression of ERβ with ERα and Progestin Receptor Proteins in the Female Rat Forebrain: Effects of Estradiol Treatment

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Estrogen and progestin receptors (ER, PgR) play a critical role in the regulation of neuroendocrine functions in females. The neuroanatomical distribution of the recently cloned, ERβ, overlaps with both ERα and PgR. To determine whether ERβ is found within ERα- or PgR-containing neurons in female rat, we used dual label immunocytochemistry. ERβ-immunoreactivity (ERβ-ir) was primarily detected in the nuclei of cells in the periventricular preoptic area (PvPO), the bed nucleus of the stria terminalis (BNSTpr), the paraventricular nucleus, the supraoptic nucleus, and the medial amygdala (MEApd). Coexpression of ERβ-ir with ERα-ir or PgR-ir was observed in the PvPO, BNSTpr, and MEApd in ovariectomized rats. E2 treatment decreased the number of ERβ-ir cells in the PvPO and BNSTpr and the number of ERα-ir cells in the MEApd and paraventricular nucleus, and therefore decreased the number of cells coexpressing ERβ-ir and ERα-ir in the PvPO, BNSTpr, and MEApd. E2 treatment increased the amount of PgR-ir in cells of the PvPO, BNSTpr, and MEApd, a portion of which also contained ERβ. These results demonstrate that ERβ is expressed in ERα- or PgR-containing cells, and they suggest that E can modulate the ratios of these steroid receptors in a brain region-specific manner. (*Endocrinology* 142: 5172–5181, 2001)

ESTROGEN (E) AND PROGESTINS are involved in the regulation of many reproductive functions (1). Although receptors for E (ER) and progestins (PgR) were discovered in the brain of female rats in the 1960s and 1970s, the more recent discovery of different subtypes and/or isoforms of these receptors has added another layer of complexity to the study of steroid signaling in the brain. In particular, the recently discovered second ER, ERβ (2–4), is expressed in the brain of a number of species (5, 6).

ERβ shares a high level of sequence identity with ERα in some receptor domains, and both receptors bind to estradiol (E2) with similar affinity (7). However, ERβ and ERα can exhibit different transcriptional activities in cotransfection experiments depending on the ligands and promoters tested (8, 9). Furthermore, ERβ and ERα have the ability to homodimerize and heterodimerize and display different transcriptional activities dependent on the particular dimer that is formed (10, 11). Thus, cellular responses to estrogenic ligands may depend in part on the presence and ratio of the ER subtypes. Therefore, to study E signaling in the brain, it is important to determine the extent of cellular coexpression of ERβ and ERα in specific neuroanatomical areas.

Recent studies have demonstrated that progesterone can alter the expression of ERβ mRNA in human breast tumors (12) and monkey corpus luteum (13). Additionally, one study showed that E2 acts through ERβ to induce PgR expression in a colon cancer cell line (14). In the brain virtually all cells in which E2 induces PgR expression contain ERα (15). However, E2 also induces some PgR expression in ERα knockout mice (ERαKO). This response could be due to the presence of residual ERα variants (16, 17). Alternatively, ERβ may mediate PgR induction upon E2 treatment in the ERαKO (17). To examine whether the presence of ERβ alters the expression of PgR in female rat brain, it is essential to first determine whether ERβ and PgR are present within the same cells.

ERβ mRNA has been detected in the brain by both RT-PCR and *in situ* hybridization (5, 7). Substantial quantities of ERβ mRNA exist in hypothalamic and limbic regions of the brain, which are known to contain high levels of ERα and PgR. ERβ mRNA has also been detected in other regions of the brain [supraoptic nucleus and paraventricular nucleus (PVN)] known to express little or no ERα or PgR (5). Recently, Shughrue *et al.* (18) also observed the presence of ERα-immunoreactivity (ERα-ir) in ERβ mRNA-containing neurons of limbic and hypothalamic regions of the female rat brain, suggesting that *in vivo*, ERα and ERβ proteins may be expressed within the same cells. ERβ protein has also been detected in rat brain by use of immunocytochemistry (19). In this study, which used an affinity-purified polyclonal antibody raised against the C-terminus of rat ERβ, ERβ protein was seen in a more limited number of cells and in fewer regions than ERβ mRNA by *in situ* hybridization.

In the present study we determined the extent of cellular coexpression of ERβ-ir with ERα-ir as well as ERβ-ir with PgR-ir in different brain regions, using a newly developed monoclonal antibody raised against a peptide from the N-terminal region of ERβ. The resulting distribution of ERβ-ir cells closely matched the distribution of cells containing moderate to high levels of ERβ mRNA in female rat brain (5). A double label immunofluorescence technique was used to

Abbreviations: BNSTpr, Bed nucleus of the stria terminalis; E, estrogen; E2, estradiol; EB, E2 benzoate; ERβ-ir, ERβ-immunoreactivity; ERαKO, ERα knockout mice; hER, human ER; MEApd, medial amygdala; OVX, ovariectomized; PgR, progestin receptor; PVN, paraventricular nucleus; PvPO, periventricular preoptic area; TBS, Tris-buffered saline.

determine whether ERβ-ir was present in ERα-ir- or PgR-ir-containing neurons in forebrain regions of ovariectomized female rats. Moreover, we tested the hypothesis that E2 treatment influenced the distribution and the ratio of coexpression of these receptors.

Materials and Methods

Animals

Female Sprague Dawley rats (175–200 g; Charles River Laboratories, Inc. Breeding Laboratories, Inc., Wilmington, MA) were group-housed for 1 wk in a 14-h light, 10-h dark cycle, with food and water available *ad libitum*. Procedures were approved by the institutional animal care and use committee of the University of Massachusetts (Amherst, MA). All rats were then ovariectomized (OVX) after ip injections of a cocktail of xylazine (5 mg/kg), ketamine (26 mg/kg), and acetopromazine (0.9 mg/kg). One week after surgeries, levels of circulating E2 are extremely low, and a bolus injection of 10 μg E2 benzoate (EB) restores them within 24 h. Thus, 1 wk later, the experimental group of females was injected sc with either 10 μg EB (n = 5) or sesame oil vehicle (0.1 ml; n = 5), and animals were killed 48 h later.

Immunoblotting

Protein extracts were from Sf21 insect cells infected without or with human (h) ERβ (1–485) or wild-type hERα recombinant virus. Cells were harvested, washed, and lysed in ice cold buffer containing 0.4 M KCl, 10 mM Tris (pH 7.5), 0.5 mM phenylmethylsulfonylfluoride, 1 μg/ml aprotinin, and 1 mM dithiothreitol. Soluble protein extracts were obtained by centrifugation at 100,000 × *g* for 45 min at 4 C. Protein concentration was determined, and extracts were added to SDS-PAGE buffer, subjected to SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Richmond, CA). Membranes were blocked in 5% Carnation instant nonfat dry milk in Tris-buffered saline (TBS; Bio-Rad Laboratories, Inc.) for 15 min. Primary hERβ antibody (1 μg/ml) was incubated with membranes overnight at room temperature in 0.5% milk in TBS/0.1% Tween-20. Goat antimouse secondary antibodies conjugated to alkaline phosphatase (Bio-Rad Laboratories, Inc.) were incubated with membranes for 2 h at room temperature in 0.5% milk/TBS/0.1% Tween-20. Washes and development of color were performed according to the Bio-Rad Laboratories, Inc., protocol.

Characterization of ERβ and ERα antibodies: ligand-bound vs. unbound receptors

To determine whether variations in ERβ or ERα immunostaining after E2 treatment may be attributable to differential recognition of ligand-bound *vs.* unbound ERs by the respective antibodies, OVX female rats were injected sc with either 50 μg 17β-E2 dissolved in 0.1 ml vehicle (n = 5) or the 50% ethanol-water vehicle (n = 5), and they were perfused 20 min later. This treatment with a large amount of free E2 for a short period of time is believed to induce maximal ligand occupancy of ERα without resulting in down-regulation of the receptor protein (20).

Control for ER down-regulation

To determine whether recognition of the antibody for the receptor is influenced by binding of EB at the same concentration as that used in the 48-h experiment and to determine whether any variations in ERα or ERβ immunostaining at 48 h could be due to ER down-regulation, a second group of female rats was injected sc with the same dose of EB as the experimental group (10 μg; n = 4) or oil vehicle (0.1 ml; n = 4), but they were perfused 2 h later, instead of 48 h later. This treatment is expected to result in higher levels of circulating E2 (21) and occupation of ERs (22) than in the 48 h experimental group, but considerably less than in the group receiving 50 μg free E2 20 min before perfusion.

Perfusions

All animals received a lethal dose of sodium pentobarbital (89 mg/kg), and they were perfused with 0.9% physiological saline (25 ml) for 1 min followed by 4% paraformaldehyde (25 ml/min) for 10 min. After the brains were removed from the cranium, they were placed into 0.1 M sodium phosphate buffer (pH 7.2) containing 20% sucrose for 48 h. Thirty-five-micron sections from the preoptic area to the midbrain region were cut on a freezing microtome, and the sections were placed into a cryoprotectant solution.

Double label immunofluorescence for ERβ-ir with either ERα-ir or PgR-ir

For all animals, two sets of sections were removed from cryoprotectant and rinsed three times for 5 min each time in 0.05 M TBS, pH 7.6. Sections were placed into a solution containing 1% H_2O_2, 20% normal goat serum, and 1% BSA for 20 min. Sections were then incubated for 2 d at 4 C in a solution containing the mouse monoclonal hERβ antibody (hERβNT-221.3, Ligand Pharmaceuticals, Inc., San Diego, CA; 1 μg/ml) raised against a synthetic peptide corresponding to the 14-amino acid N-terminal sequence of hERβ (1–485 form) and either the rabbit polyclonal rat ERα antibody (C1355; gift from M. Shupnik, University of Virginia, Charlottesville, CA; 1:30,000) raised against the last 14-amino acid C-terminal sequence of rat ERα or the rabbit polyclonal human PgR antibody (DAKO Corp., Carpinteria, CA; 1:500) raised against a synthetic peptide corresponding to the DNA-binding domain of human PgR (PgR-A and PgR-B forms; 533–547), in a buffer containing TBS, 0.1% gelatin, 0.02% sodium azide, 0.5% Triton X-100, and 1% normal goat serum (TBS-gel). After three washes in TBS-gel buffer, the sections were incubated in a solution containing the cyanine goat antimouse (7 μg/ml, to reveal ERβ-ir) and the fluorescein goat antirabbit (10 μg/ml, to reveal ERα-ir or PgR-ir) secondary antisera for 90 min at room temperature. The sections were then rinsed three times for 5 min each time in TBS, mounted onto slides, air-dried, and coverslipped with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA).

Immunocytochemistry controls

ERβ-ir preadsorption. To determine the specificity of the anti-ERβ antibody immunostaining for ERβ, the anti-ERβ antibody was preadsorbed at 4 C overnight with a 100-fold molar excess of its corresponding synthetic peptide before use in the immunostaining assays and was used to immunostain brain sections.

PgR-ir preadsorption. To determine the specificity of the PgR immunostaining, the rabbit anti-PgR antibody was preadsorbed at 4 C overnight with a 100-fold molar excess of a mix of human PgR-A and PgR-B recombinant proteins (Tissue Culture Core Facility of the University of the Colorado Cancer Center) before use in immunostaining.

Double label immunofluorescent controls. To control for nonspecific immunofluorescent staining, cross-immunoreactivity, and "bleed-through" of the fluorochromes, sections were also incubated either in solutions in which the primary antibodies were omitted and the secondary antibodies were present or in solutions in which only one primary antibody was present and both secondary antibodies were present. No nonspecific immunofluorescent staining, cross-immunostaining, or bleed-through was observed (data not shown).

Data analysis

Quantification of double labeled immunofluorescent cells. Quantifications were performed on one matched section per brain region for each rat. Using the NIH Image computer analysis system (developed at the NIH and available at http://rsb.info.nih.gov/nih-image/), digitized pictures of the same microscopic field were captured with two different bandpass filters, specific for cyanine and fluorescein. The images were superimposed, and the numbers of single or double labeled cells were counted by eye in the brain region chosen. Because the main point of the present study was to report the level of colocalization of ERβ with either ERα or PgR, the regions examined were selected based upon the presence of ERβ-ir cells, that is, in the principal nucleus of the bed nucleus of the stria terminalis (BNSTpr), the periventricular zone of the preoptic area (PvPO), the posterodorsal nucleus of the medial amygdala (MEApd), and PVN. The quantification for all groups was performed with a ×10 objective for each brain region (BNSTpr, 340 × 300 μm; PvPO, 580 × 120 μm; MEApd, 870 × 240 μm; PVN, 390 × 480 μm), except for the 2 h

control groups, in which the quantification was performed with a ×20 objective (BNSTpr, 180 × 160 μm; PvPO, 420 × 90 μm; MEApd, 370 × 160 μm). In all microscopic fields observed, only cells that were clearly distinguishable were counted.

Variation in PgR immunofluorescent intensity in cells of oil- and EB-treated females. Many studies have shown that variations in immunostaining intensity can be measured by variations in OD (mean pixel densities) (23–25). Using inverted digitized pictures for PgR-ir, the mean pixel density of PgR-ir was measured in the PvPO, BNSTpr, MEApd, and PVN in oil- and EB-treated females, as described in detail previously (25). In brief, the gain and black levels of the camera were set so that the gray level ranged from 0 to 255 pixel density for PgR immunoreactivity (0 being white and 255 being black). The density threshold was set to determine the contribution of the background immunoreactivity and to exclude it from the contribution of the foreground immunoreactivity. The OD of PgR-ir (*i.e.* the intensity of PgR immunofluorescence) was determined for each area and was represented by the average and SEM of the pixel densities of the sections analyzed.

Variation in PgR immunofluorescent intensity in cells coexpressing ERβ-ir. PgR-ir was apparent in many cells with or without EB treatment. However, the OD of PgR-ir in EB-treated females was considerably brighter (see above, Table 3). Therefore, to determine whether cells in which EB treatment increased PgR expression contained ERβ-ir, the darkly immunostained PgR neurons were counted in EB-treated females using a method similar to that described above. The density threshold was set to eliminate E2-independent PgR immunoreactivity in oil-treated females, and was used to measure foreground PgR immunoreactivity in EB-treated females. Processed images were superimposed on corresponding digitized pictures containing ERβ-ir, and the darkly immunostained PgR neurons coexpressing ERβ-ir were counted by eye in the PvPO, BNSTpr, and MEApd.

Statistical analysis. *t* tests with $P < 0.05$ were used to determine statistical difference in the mean number of labeled cells per brain regions and in the mean percent colocalization of steroid receptors in labeled cells per brain regions.

Results

Specificity of ERβ antibody

Immunoblotting was used to test the specificity of the ERβ antibody used in the immunostaining studies. An immunoreactive band of the predicted molecular mass of ERβ (~55 kDa) was observed on blots probed with the ERβ antibody in lanes containing protein extract from insect cells infected with hERβ recombinant virus, but not in lanes containing extracts from mock- or hERα-infected cells (Fig. 1A). Therefore, this antipeptide ERβ monoclonal antibody reacts with ERβ, but not with ERα, on a Western blot. Additionally, this antibody reacted with ligand-occupied ERβ, but not with holo-ERα, as assessed by ligand binding immunoprecipitation of the recombinant proteins (data not shown).

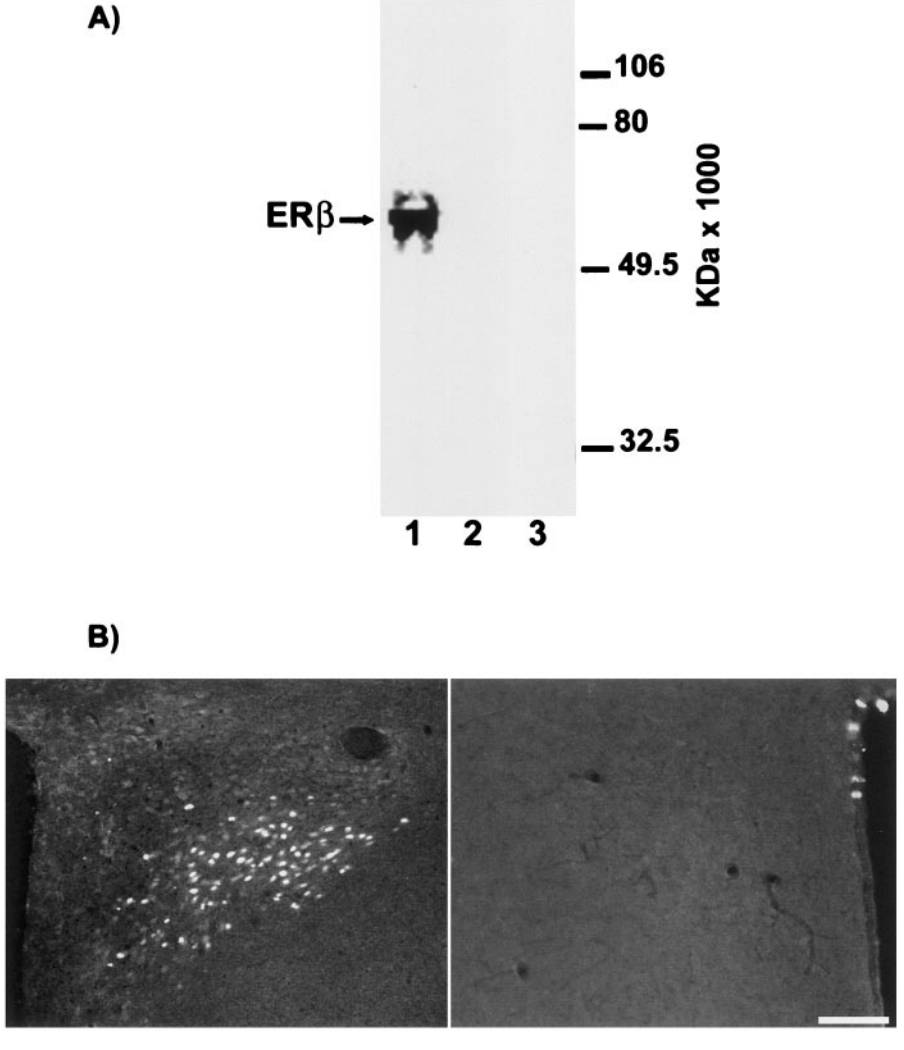

FIG. 1. A, Immunodetection of hERβ, but not hERα, with the anti-ERβ monoclonal antibody. Total soluble protein extracts (10 μg) from noninfected Sf21 cells (lane 3) or from Sf21 cells infected with hERβ-(1–485) (lane 1), or hERα (lane 2) were electrophoresed via SDS-PAGE and transferred to nitrocellulose membranes. After blocking, the membranes were incubated with ERβ monoclonal antibody as described in *Materials and Methods*. B, ERβ immunostaining in the PVN. Photomicrographs of the PVN immunostained with the N-terminus hERβ antibody (*left panel*) and the N-terminus hERβ antibody preadsorbed with its specific peptide (*right panel*) in OVX, oil-treated females. *Scale bar*, 60 μm.

Characterization of ERβ and ERα antibodies: ligand-bound vs. unbound receptors

As some steroid receptors antibodies are preferential in their binding to either the unoccupied (20) or the occupied (26) form of the receptor, we compared the number of ERα-ir and ERβ-ir cells after a 20-min injection of 50 μg 17β-E2. The number of ERα-ir cells did not vary in any brain region 20 min after the injection of 50 μg free 17β-E2 (vehicle *vs.* E_2: in PvPO, 116 ± 10.5 *vs.* 116.2 ± 5.2; in BNSTpr, 178 ± 15.1 *vs.* 178.2 ± 16.8; in MEApd, 177.6 ± 8.1 *vs.* 178.6 ± 18.6), demonstrating that the polyclonal ERα antibody used in the study recognizes both ligand-bound and unbound ERα. In contrast, immunoreactivity for ERβ was not detectable 20 min after treatment with 50 μg free 17β-E2 (vehicle *vs.* E_2: in PvPO, 43.2 ± 6 *vs.* 0 ± 0; in BNSTpr, 54 ± 11.5 *vs.* 0 ± 0; in MEApd, 77.8 ± 4.6 *vs.* 0 ± 0), suggesting that ERβ immunostaining is greatly affected by occupation of the receptor with a saturating dose of free E2.

Neuronal distribution of ERα-ir, ERβ-ir, and PgR-ir, and their regulation by EB treatment

The pattern of distribution of ERα-ir in oil- or EB-treated female brains was consistent with previous reports (27–29). ERα-ir was present in nuclei of neurons of the preoptic area; the PvPO; the principal and medial nuclei of the BNST; the anterior, lateral and ventromedial hypothalamus; the parvocellular part of the PVN; the central, medial, and cortical amygdala; the arcuate nucleus; the piriform cortex; and, to a lesser extent, the hippocampus. The number of ERα-ir neurons in the MEApd and PVN significantly decreased 48 h after EB treatment ($P < 0.05$; Table 1). The number of ERα-ir cells did not vary in any brain region 2 h after treatment with 10 μg EB *vs.* treatment with vehicle (oil *vs.* EB: in PvPO, 106.6 ± 10.8 *vs.* 128.3 ± 5.2; in BNSTpr, 208 ± 7.9 *vs.* 181.4 ± 40; in MEApd, 185.6 ± 23 *vs.* 164 ± 24.6), consistent with the finding that occupancy of ERα by E2 does not change the interaction of the ERα antibody, C1355, with ERα. Therefore, the decrease in ERα-ir cells observed at 48 h is probably due to down-regulation of ERα by E2.

TABLE 1. Coexpression of ERα and ERβ in brain regions

Area	Labeling	Oil	EB
BNSTpr	ERα-ir	377.8 ± 23.1	365.8 ± 60.5
	ERβ-ir	128.4 ± 22.3	50.8 ± 10.6[a]
	ERα-ERβ-ir	80.7 ± 5.9	52.8 ± 5.2[a]
PvPO	ERα-ir	219.4 ± 17.7	189.2 ± 15.5
	ERβ-ir	97.0 ± 12.4	29.2 ± 2.8[a]
	ERα-ERβ-ir	90.8 ± 14.1	25.5 ± 2.0[a]
MEApd	ERα-ir	549.0 ± 24.0	361.8 ± 59.9[a]
	ERβ-ir	129.0 ± 20.6	123.4 ± 24.8
	ERα-ERβ-ir	90.0 ± 14.0	38.2 ± 9.3[a]
PVN	ERα-ir	72.6 ± 16.1	35.2 ± 7.1[a]
	ERβ-ir	96.0 ± 5.7	93.8 ± 10.9
	ERα-ERβ-ir	3.4 ± 1.3	2.2 ± 1.3

The numbers (mean ± SEM) of ERα-ir-, ERβ-ir-, and ERα-ERβ-ir-containing neurons (cells per unit area; see *Materials and Methods* for area of each brain region) in the BNSTpr, PvPO, MEApd, and PVN of OVX rats treated for 48 h with oil (n = 5) or EB (n = 5) were counted as described in *Materials and Methods*.

[a] Significantly different from oil group (by *t* test, $P < 0.05$).

The distribution of ERβ-ir cells was consistent with the expression patterns reported for ERβ mRNA (5) and protein (19). The hERβ antibody used in this study specifically recognized recombinant ERβ protein by immunoblotting (Fig. 1A), and the immunostaining with this antibody was completely blocked by preadsorption of the antibody with the peptide that it was generated against (Fig. 1B). In the forebrain, ERβ-ir was found in nuclei of cells in the PvPO, the dorsal and ventral BNSTpr, the dorsal and magnocellular part of the PVN, the anterior and caudal supraoptic nucleus, and the anterior and posterodorsal MEA (Fig. 2). The number of ERβ-ir cells significantly decreased in the PvPO and BNSTpr 48 h after EB treatment *vs.* treatment ($P < 0.05$; Tables 1 and 2). Even though the level of E2-occupied ERβ is probably higher at 2 h than at 48 h, the number of ERβ-ir cells did not change in any brain region 2 h after treatment with 10 μg EB *vs.* treatment with vehicle (oil *vs.* EB: in PvPO, 49.3 ± 13 *vs.* 59.3 ± 15; in BNSTpr, 69 ± 4.9 *vs.* 63.3 ± 9.5; in MEApd, 91.3 ± 10 *vs.* 77.6 ± 20). This suggests that the dose of EB (10 μg) used in the experiment did not interfere with antibody binding to the receptor, even though a much larger dose of free E2 (50 μg) interfered with immunostaining. Therefore, the decrease in ERβ-ir seen at 48 h represents down-regulation of ERβ-ir by E2.

The distribution of PgR-ir in the brain of both oil- and EB-treated female rats was more extensive than previously reported (25, 30–32), because of greater sensitivity of the immunostaining technique used here. No immunostaining was observed with PgR-preadsorbed PgR antibodies (data not shown). Many PgR-ir cells were detected in the preoptic area, BNST, the lateral and ventromedial hypothalamus, the arcuate nucleus, and, to a lesser extent, the parvocellular part of the PVN. PgR-ir was also observed in the hippocampus, the piriform cortex, various amygdala nuclei, the magnocellular part of the PVN, and layers III–VI of the cerebral cortex (data not shown). The number of PgR-ir neurons in the PvPO, the BNSTpr, the MEApd, and the PVN did not differ between oil- and EB-treated females (Table 2). However, E2 treatment resulted in a significant increase ($P < 0.05$) in staining intensity (*i.e.* pixel density) of PgR-ir in the PvPO, BNSTpr, and MEApd of EB-treated females (Table 3), consistent with previous findings that there is E2-independent PgR expression throughout the brain, and that E2 can also increase PgR levels.

Coexpression of ERα-ir and ERβ-ir

Colocalization of ERα-ir and ERβ-ir was observed in cells of the PvPO, the BNSTpr, and the MEApd (Table 1 and Fig. 3). The number of neurons expressing both ERα-ir and ERβ-ir decreased significantly after EB treatment in the PvPO, BNSTpr, and MEApd (Table 1). The percentage of ERα-ir neurons containing ERβ-ir in the PvPO and MEApd decreased significantly in the EB-treated group compared with the oil-treated group ($P < 0.05$; Fig. 4A). Conversely, the percentage of ERβ-ir neurons containing ERα-ir in the MEApd decreased significantly in the EB-treated group compared with the oil-treated group ($P < 0.05$; Figs. 4B). In the PVN, because ERα-ir and ERβ-ir were in anatomically distant sites, very

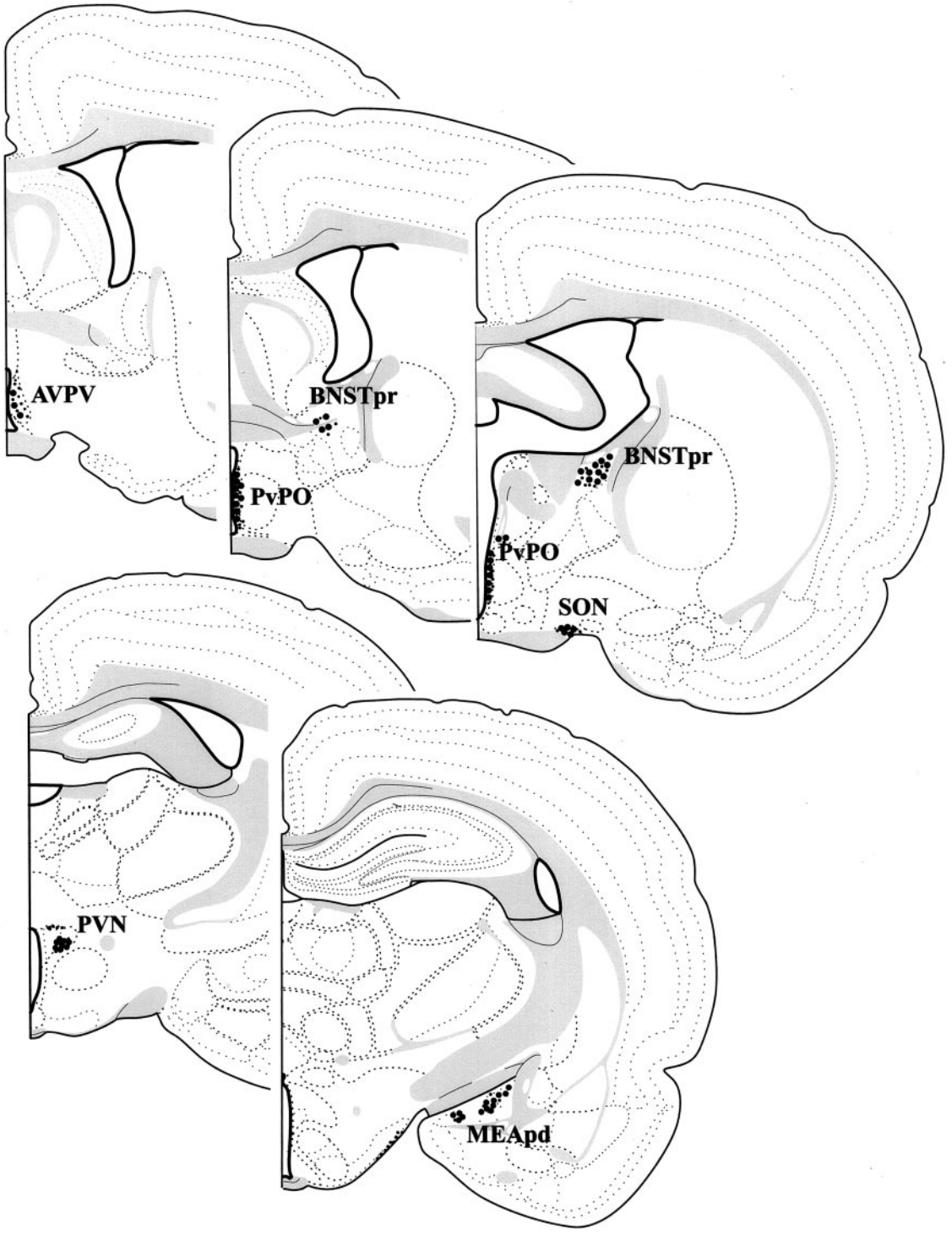

FIG. 2. Distribution of ERβ-ir cells in female brain. AvPV, Anteroventral periventricular nucleus; SON, supraoptic nucleus. One *small dot* represents 1 ERβ-ir cell; one *large dot* represents 10 ERβ-ir cells.

little colocalization of ERα-ir and ERβ-ir was observed (Table 1 and Fig. 4, A and B).

Coexpression of PgR-ir and ERβ-ir

Coexpression of PgR- and ERβ-ir was observed in cells of the PvPO, the BNSTpr, the MEApd, and the PVN (Table 2). The number of neurons coexpressing both PgR-ir and ERβ-ir in all regions observed did not differ between groups (Table 2). The percentage of PgR-ir neurons containing ERβ-ir in the PvPO decreased significantly in the EB-treated group compared with the oil-treated group ($P < 0.05$, Fig. 5A). In all regions observed, there was no difference in the percentage of ERβ-ir neurons containing PgR-ir between groups (Fig. 5B).

As in this experiment the PgR immunocytochemical pro-

cedure used was more sensitive than in previous work, ER-independent PgR were also detected. To focus only on the E2-induced PgR expression, we adjusted our criterion of imaging PgR to eliminate ER-independent PgR-ir (see *Materials and Methods*). Upon counting only darkly immunostained PgR cells in the EB-treated group by selecting an upper threshold for pixel density as a mean of selecting only E2-induced PgR-ir cells, it was observed that about 35%, 28%, and 25% of PgR-ir neurons coexpressed ERβ-ir in the PvPO, the BNSTpr, and the MEApd, respectively (Fig. 6).

Discussion

In the forebrain of female rats, ERβ-ir was coexpressed in ERα-ir- and PgR-ir-containing neurons. Furthermore, E2 treatment modulated the expression of ERβ, ERα, and PgR in a brain region-specific manner. This caused a change in the ratio of cells coexpressing both ER subtypes or ERβ and PgR in specific brain areas. Also as expected, E2 treatment induced the expression of PgR in hypothalamic and limbic regions, which occurred in part in ERβ-ir-containing neurons.

The hERβ antibody used in the present study appears to be very specific for ERβ as assessed by immunoblotting and immunoprecipitation assays. The antibody reacted with ERβ, but not ERα, PgR, or AR, by Western blot (Fig. 1A and data not shown) and by the elimination of immunostaining after antibody preadsorption with its specific peptide (Fig. 1B). Although the antibody recognized the ligand-bound ERβ by *in vitro* immunoprecipitation assays using recombinant proteins (data not shown), we found that sc treatment of ovariectomized female rats with a large amount of free E2 (50 μg) eliminated ERβ immunostaining in all brain regions. This suggests that in brain tissue, this antibody does not react with ligand-bound ERβ. Discrepancies between *in vitro* and *in vivo* characteristics of antibodies have previously been reported, such as for the specific ERα antibody, H222 (20). Although antibodies raised against the N-terminus of ERα were not reported to show differential interactions with ERα (20), this may not be the case for the ERβ antibody used in these studies. Blocking of the ERβ antibody epitope by ligand binding may occur *in vivo* through conformational changes in ERβ upon binding of ligand, by dimerization of the receptor with ERα or ERβ, or by interaction of ERβ with other accessory proteins, such as cofactors.

The distribution of ERβ-ir (Fig. 2) as assessed with the N-terminus hERβ monoclonal antibody was different from that in previous studies in which a polyclonal C-terminal rat ERβ antibody was used (19, 33). Using the C-terminal ERβ antibody, fiber and cytoplasmic stainings were observed in the lateral septum and the hippocampus (19), which were not observed using the N-terminus ERβ antibody. In contrast to the C-terminal antibody, which labeled cells in the lateral septum, the medial BNST, the PVN, the supraoptic nucleus, the anterior MEA, and the hippocampus (19), the N-terminal antibody labeled numerous cells within the PvPO, the BNSTpr, the PVN, the supraoptic nucleus, and the MEApd, where the highest density of ERβ mRNA-containing cells and the highest level of ERβ mRNA have been reported (5). This suggests that the human N-terminus ERβ antibody used in the present study may specifically recognize cells containing moderate to high levels of ERβ. Thus, the extent of colocalization of ERβ with ERα or PgR and the changes in the ratio of steroid-containing cells after hormonal treatment reported here may be underestimated. These should, of course, be taken as relative indexes of colocalization and not as absolute numbers. It is also possible that ERβ mRNA is expressed at a higher level than ERβ protein. Differential protein expression of ERβ variants that have been reported in the brain (34) could also be an explanation for the differences in ERβ distribution observed between *in situ* hybridization and immunocytochemical approaches.

Little work has been reported on the hormonal regulation of ERβ in the brain. Previous studies reported that E2 treatment caused down-regulation of ERβ mRNA expression in the PVN (35) and the MEA (36). Although we did not observe a decrease in the number of ERβ-ir cells in the PVN or the MEApd 48 h after EB injection, the number of ERβ-ir cells decreased in the PvPO and the BNSTpr. This decrease is probably due to down-regulation of ERβ in specific brain regions. Although an injection of 50 μg free E2 20 min before perfusion interfered with ERβ antibody binding to ERβ, an injection of 10 μg E2 benzoate 2 h before perfusion resulted in apparent levels of ERβ-ir not different from those observed

TABLE 2. Coexpression of PgR and ERβ in brain regions

Area	Labeling	Oil	EB
BNSTpr	PgR-ir	162.8 ± 14.5	184.0 ± 22.5
	ERβ-ir	154.6 ± 23.6	74.8 ± 16.3[a]
	PgR-ERβ-ir	51.6 ± 15.0	37.2 ± 10.0
PvPO	PgR-ir	144.0 ± 26.7	181.6 ± 23.6
	ERβ-ir	92.4 ± 9.8	49.0 ± 16.1[a]
	PgR-ERβ-ir	72.2 ± 10.6	46.0 ± 17.3
MEApd	PgR-ir	184.6 ± 26.3	225.8 ± 41.8
	ERβ-ir	129.2 ± 7.6	94.2 ± 23.1
	PgR-ERβ-ir	24.8 ± 4.4	24.0 ± 3.2
PVN	PgR-ir	154.0 ± 17.3	137.0 ± 20.9
	ERβ-ir	93.0 ± 9.6	80.4 ± 17.8
	PgR-ERβ-ir	65.4 ± 4.6	50.0 ± 6.1

The numbers (mean ± SEM) of PgR-ir-, ERβ-ir-, and PgR-ERβ-ir-containing neurons (cells per unit area; see *Materials and Methods* for area of each brain region) in the BNSTpr, PvPO, MEApd, and PVN of OVX rats treated with oil (n = 5) or EB (n = 5) were counted as described in *Materials and Methods*.

[a] Significantly different from oil group (by *t* test, $P < 0.05$).

TABLE 3. E2 treatment increases PgR expression in brain regions

	BNSTpr	PvPO	MEApd	PVN
Oil	1,722.4 ± 194	6,765.8 ± 1,244	4,380.6 ± 265	3,452.2 ± 145
EB	5,300.4 ± 438[a]	24,067.8 ± 1,635[a]	5,285.2 ± 189[a]	3,739.8 ± 512

Pixel density per area (mean ± SEM) for PgR-ir labeled cells in the BNSTpr, PvPO, MEApd, and PVN of oil- (n = 5) or EB-treated OVX female rats (n = 5).

[a] Significantly different from oil group (by *t* test, $P < 0.05$).

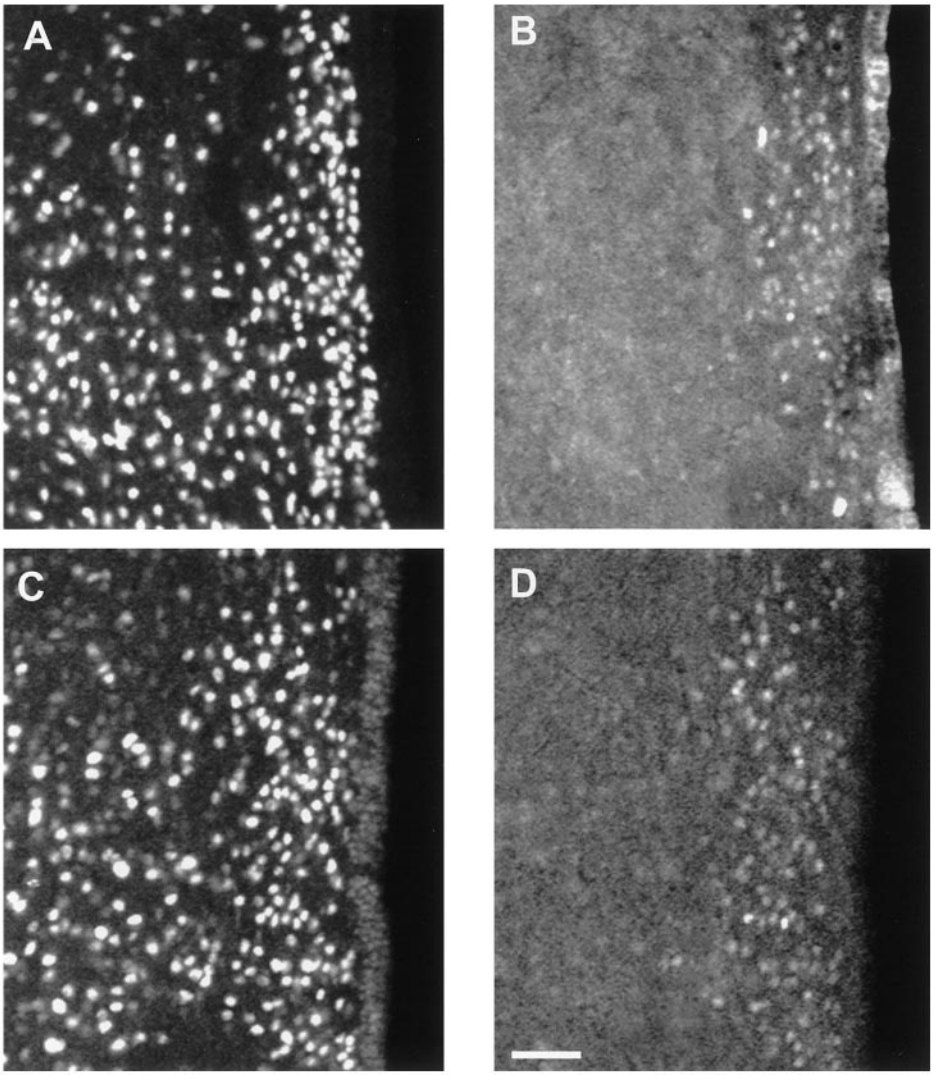

FIG. 3. ERα, ERβ, and PgR immunostaining in PvPO. Photomicrographs in the PvPO of OVX, EB-treated females of the same microscopic fields showing ERα-ir (A), and ERβ-ir (B), PgR-ir (C), and ERβ-ir (D). *Scale bar*, 60 μm.

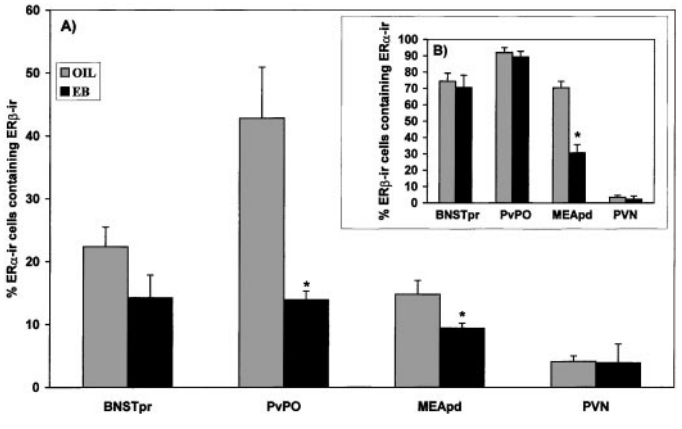

FIG. 4. Colocalization of ERα-ir and ERβ-ir in brain areas. Percent of ERα-ir cells containing ERβ-ir (A) and percentage of ERβ-ir cells containing ERα-ir (B) in the BNSTpr, the PvPO, the MEApd, and the PVN of OVX, oil-treated (▨) and EB-treated (■) females. *, Significantly different from the oil group (by *t* test, $P < 0.05$).

after vehicle treatment. This suggests that even though ERβ immunostaining can be influenced by occupation of the receptor with a saturating dose of free E2 (50 μg), exposure to a low level of circulating E2 (10 μg EB) does not interfere with the ERβ immunostaining. Thus, decreases in ERβ immunostaining observed after EB injection are probably due to down-regulation of ERβ. Although this issue needs to be further examined with molecular approaches, the present results are consistent with reports of down-regulation of ERβ mRNA in the brain (35, 36) and other tissues (37) after E2 treatment. Therefore, these findings suggest that ERβ protein expression may be differentially regulated by E2 in various regions of the brain.

Our observations on the presence of ERα-ir in hypothalamic and limbic regions, including the PvPO, the BNSTpr, the parvocellular part of the PVN, and the MEApd, agree with previous reports using various ERα antibodies (27–29). E2 treatment significantly decreased the number of ERα-ir neurons in the MEApd and the PVN. This decrease is also probably due to a brain region-specific down-regulation of ERα, as ligand binding did not influence ERα-ir staining (with the C1355 antibody used in this study) in any brain region. Moreover, these findings agree with previous reports of down-regulation of ERα or ERα mRNA in the brain after E2 administration (38, 39).

In vitro studies suggest that ERβ and ERα may het-

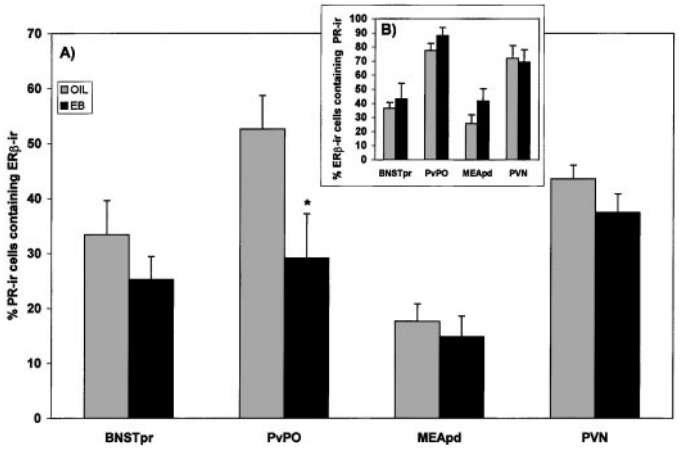

FIG. 5. Colocalization of PgR-ir and ERβ-ir in brain regions. Percentage of PgR-ir cells containing ERβ-ir (A) and percentage of ERβ-ir cells containing PgR-ir (B) in the BNSTpr, the PvPO, the MEApd, and the PVN of OVX, oil-treated (▨) and EB-treated (■) females. *, Significantly different from the oil group (by *t* test, $P < 0.05$).

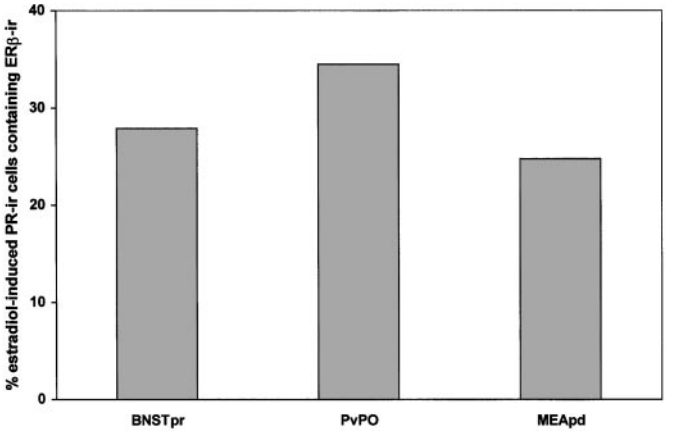

FIG. 6. Colocalization of PgR-ir of high immunofluorescent intensity with ERβ-ir-containing cells in the BNSTpr, the PvPO, and the MEApd of OVX, EB-treated females.

erodimerize (10). For this heterodimerization to occur in the brain, both ERα and ERβ must be expressed in the same neurons. Colocalization of ERα-ir and ERβ-ir was observed in neurons of the PvPO, the BNSTpr, the MEApd, as well as the PVN. Depending on the region and the hormonal treatment, this represented less than 40% of the ERα-containing cell population, but this represented over 60% of the ERβ-ir neurons, except in the PVN where very little colocalization was observed. Shughrue *et al.* (18) previously reported a comparable, high level of ERβ cells coexpressing ERα in the PvPO, the BNST, and the MEApd when examining the colocalization of ERβ mRNA and ERα-ir. In contrast in the present study because the number of ERβ-ir cells is lower than the number of ERβ mRNA cells in the brain regions observed, the levels of ERα-containing cell coexpressing ERβ are lower than those reported in the earlier study (18). Nevertheless, these findings provide evidence that ERα and ERβ have the opportunity to interact *in vivo* intracellularly in a small population of limbic and hypothalamic neurons. Furthermore, it suggests that in these brain regions, transcriptional regulation by E2 could occur via at least three classes of cells: cells containing ERα, ERβ, or both.

Because ERβ expression decreased in the PvPO after E2 treatment, the number of ERα neurons containing ERβ decreased significantly. This decrease in neurons coexpressing both ERs also results in an increase in the number of cells expressing only ERα-ir. In contrast to the E2-induced decrease in ERβ in the PvPO, in the MEApd ERα expression significantly decreased after E2 treatment, which induced an increase in the number of cells expressing ERβ only. As previously mentioned, ERα and ERβ isoforms have the ability to form heterodimers (10, 34), which have different transcriptional activities than ER homodimers (11). Together with the present data, this further suggests that differential regulation of ERs by E2 may lead to cells with different profiles of E responsiveness in a brain region-specific manner.

The neural distribution of PgRs in females and the increase in its expression by E2 in some brain regions have been described extensively using a variety of techniques (30, 31, 40). In other immunocytochemical studies, a limited distribution of PgR-ir has been observed in the brain due to the sensitivity of the immunocytochemical procedure adjusted so that either only cells in which E2-induced PgR-ir were labeled (30) or only dark nuclear staining was observed (32). Using a higher concentration of antibody than had been used previously, we detected PgR-ir in neurons of regions previously described as containing E2-induced PgR, such as in the PvPO, the preoptic area, the BNSTpr, the lateral ventromedial hypothalamus, the arcuate nucleus and the MEApd, as well as E2-independent PgR such as in the PVN and the cerebral cortex (31, 41–43). The requirement of a higher concentration of antibody necessary to visualize PgR in the PVN and the cortex suggests a lower abundance of the receptor in these regions. Both isoforms of PgR (PgR-A and PgR-B) have been detected in the brain (41). Although it has been suggested that the expression of one of the two PgR isoforms (PgR-B) has less dependence on E2 (41), the PgR antibody used here recognizes both PgR isoforms and does not allow us to discriminate the two. In accordance with previous reports (30), E2 injection increased the expression of PgR-ir, as seen in the present study by an increase in optical intensity in the PvPO, BNSTpr, and MEApd, but no effect of E2 on PgR expression was detected in the PVN. This finding agrees with earlier ligand binding studies in which progesterone binding was seen throughout the brain, but E2 treatments increased PgR expression only in limited areas (44).

Although the coexpression of PgR and ERβ has been reported in cell lines and human tumor cells (12, 45), we report here the *in vivo* coexpression of PgR-ir and ERβ-ir in neurons of the PvPO, the BNSTpr, the MEApd, and the PVN of female rat brains. Depending on the region and the hormonal status of the females, 20–50% of the PgR-ir cells coexpressed ERβ-ir. Conversely in the PvPO and PVN, over 70% of the ERβ-ir cells coexpressed PgR-ir, whereas only about 45% did in the BNSTpr and the MEApd. It is well known that E2 induces the expression of PgR in ERα-containing cells of the female brain (15, 46). In the present study E2-induced PgR-ir was measured by an increase in the intensity of PgR-ir. It is important to note that ERβ-ir was found in cells containing E2-induced PgR-ir (EB-treated) as well as cells containing E2-indepen-

dent PgR-ir (*i.e.* in oil-treated rats) in all regions tested. More specifically, about 30% of cells that showed an increased expression of PgR-ir coexpressed ERβ-ir. We did not test for the presence of ERα in these cells. However, as mentioned earlier, E2-induced PgR-ir is almost exclusively in ERα-containing cells (15), strongly supporting the idea that cells with E2-induced PgR and ERβ-ir may also contain ERα.

In ERα-gene disrupted (ERαKO) mice, E2 treatment increased the expression of PgR mRNA in the PvPO (16) and of PgR-ir in the MEApd as well as in the arcuate nucleus and the lateral ventromedial hypothalamus (17). Although our present data suggest the involvement of both ERα and ERβ in the regulation of PgR expression, future studies using various steroid receptor gene-disrupted models will be needed to further examine whether the induction of PgR expression by E2 is mediated by ERα variants, ERβ, or both.

It has recently been shown that ERβ plays a modest role, if any, in the regulation of female sexual behavior. Indeed, although sexual behavior is not impaired in ERβ gene-disrupted mice, the duration of sexual receptivity in females is extended compared with that in wild-type animals (47). On the other hand, the brain regions in which ERβ-ir is present are known to be involved in gonadal hormone regulation of a variety of neuroendocrine systems. For example, the posterior medial BNST (level equivalent to the BNSTpr) is involved in the regulation of the CRH expression in the PVN (48), the expression of which is regulated by gonadal hormones. Likewise, in the supraoptic nucleus and PVN, ERβ has been located in oxytocin- and vasopressin-containing cells (33), which are involved in reproductive functions.

Our results demonstrated that ERβ is present in cells containing other steroid receptors in brain regions that are of importance in females, and they suggest that ERβ alone or in combination with ERα and/or PgR may participate in the hormonal regulation of a variety of neuroendocrine functions. Furthermore, our results suggest that E2 may play a crucial role in regulating amounts of ERs (α and β) and PgRs in cells in a brain-region specific manner, which may, in turn, induce differential expression of relevant genes.

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