Distribution and Estrogen Regulation of Membrane Progesterone Receptor-β in the Female Rat Brain

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Although several studies have reported the localization of membrane progesterone (P_4) receptors (mPR) in various tissues, few have attempted to describe the distribution and regulation of these receptors in the brain. In the present study, we investigated expression of two mPR subtypes, mPRα and mPRβ, within regions of the brain, known to express estradiol (E_2)-dependent [preoptic area (POA) and hypothalamus] and independent (cortex) classical progestin receptors. Saturation binding and Scatchard analyses on plasma membranes prepared from rat cortex, hypothalamus, and POA demonstrated high-affinity, specific P_4-binding sites characteristic of mPR. Using quantitative RT-PCR, we found that mPRβ mRNA was expressed at higher levels than mPRα, indicating that mPRβ may be the primary mPR subtype in the rat brain. We also mapped the distribution of mPRβ protein using immunohistochemistry. The mPRβ-immunoreactive neurons were highly expressed in select nuclei of the hypothalamus (paraventricular nucleus, ventromedial hypothalamus, and arcuate nucleus), forebrain (medial septum and horizontal diagonal band), and midbrain (oculomotor and red nuclei) and throughout many areas of the cortex and thalamus. Treatment of ovariectomized female rats with E_2 benzoate increased mPRβ immunoreactivity within the medial septum but not the medial POA, horizontal diagonal band, or oculomotor nucleus. Together, these findings demonstrate a wide distribution of mPRβ in the rodent brain that may contribute to functions affecting behavioral, endocrine, motor, and sensory systems. Furthermore, E_2 regulation of mPRβ indicates a mechanism through which estrogens can regulate P_4 function within discrete brain regions to potentially impact behavior. **(*Endocrinology* 153: 4432–4443, 2012)**

The ovarian steroid hormones estradiol (E_2) and progesterone (P_4), regulate cellular functions in the central nervous system (CNS), thereby altering reproductive physiology and behaviors in female rodents (1–3). The regulatory action of E_2 involves activation of estrogen receptors in the ventromedial hypothalamus and the preoptic area (POA), which in turn act as ligand-dependent transcription factors and alter the expression of genes, including the progestin receptor [Pgr; P_4 receptor (PR)]. The time course of activation and termination of reproductive behavior parallels E_2-induced increase and decline in Pgr in the ventromedial hypothalamus and the POA of the brain (4–7). Although genomic effects, characterized by a delayed onset, have traditionally been assumed to be the primary pathway for P_4 effects on female reproductive behavior, reports suggest the involvement of nonclassical mechanisms in this function. These nonclassical, short-latency effects of P_4 may be mediated through the modulation of putative cell surface receptors, ion channels, and mechanisms coupled to cytoplasmic second messenger signaling cascades, independent of gene transcription (8–11). Interactions between membrane-initiated P_4 effects

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Abbreviations: CNS, Central nervous system; E_2, estradiol; ER, E_2 receptor; ir, immunoreactive; mPR, membrane PR; NGS, normal goat serum; P_4, progesterone; Pgr, progestin receptor; Pgrmc, PR membrane component; POA, preoptic area; PR, P_4 receptor; TBS, Tris-buffered saline.

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and intracellular classical Pgr have been observed in the facilitation of reproductive behavior in female hamsters (12, 13), suggesting that both classical and nonclassical mechanisms act in concert rather than independently.

Rapid, nonclassical actions of P_4 have been shown to occur within the CNS and initiate and/or sustain physiological responses and reproductive behavior (4, 14–18). These short-latency effects of P_4 modulate a plethora of cell functions including the release of LHRH (19) and dopamine and acetylcholine (20), release of excitatory amino acids (21), changes in neuronal activity (22, 23), and activation of intracellular signaling cascades in rats and mice (16–18, 24, 25). However, the molecular mechanisms mediating these rapid actions remain elusive. Multiple subtypes of membrane PR (mPR) have been described in vertebrates (26, 27). Three of these receptors, designated mPRα (*PAQR7*), mPRβ (*PAQR8*), and mPRγ (*PAQR5*), have a high binding affinity for P_4 and appear to play a role in reproduction in fish, amphibians and rodents (28–30), although their function in mammalian species is not well described. The mPR belong to the progestin and adipoQ receptor (*PAQR*) family (31, 32) and mediate rapid progesterone actions through G protein activation and alterations in intracellular signaling pathways.

Various mPR subtypes have been identified in a wide variety of tissues including reproductive organs and the CNS (31, 33–35). Human mPRα, -β, and -γ show some differences in their tissue distributions with high levels of mPRα in the testis, ovary, and placenta, indicating the α-subtype may mediate reproductive functions (26). Similarly, mPRα has been identified as a likely mediator of progestin-induced increases in sperm motility and oocyte maturation (26, 29). Sleiter *et al.* (30) have demonstrated the presence of mPRα and mPRβ message in the medial basal hypothalamus and their potential involvement in the negative feedback effects of P_4 on GnRH secretion. Recent studies have also demonstrated mPRα in the other brain regions as well as the spinal cord of rodents (33, 34). mPRβ is highly expressed in human neural tissue (26) and has also been detected within the mouse and rat brain and mouse spinal cord (32–34). The expression of human mPRγ is high within the human kidney, intestine, and lung (31), whereas in rodents, it has also been localized within the ovary, fallopian tube, lung, and liver (36), and its expression in the rodent spinal cord is low (33).

In rodents, E_2 priming results in an increase in expression of the classical nuclear Pgr in the hypothalamus and POA but not in the cerebral cortex (37, 38). These increases in expression appear largely mediated by activation of E_2 receptor (ER)-α (39, 40). The number of E_2-inducible Pgr in the hypothalamus and the POA is maximal at 48 h (4), around the time of preovulatory P_4 release, and parallels the time of activation of reproductive behavior in rodents (4, 6, 37, 41, 42). Recent studies indicate that mRNA levels of mPR, mPRα, and mPRβ, but not of mPRγ, are also elevated in the mediobasal hypothalamus on the afternoon of proestrus, around the time of the preovulatory peak of P_4 in cycling rats (43). The findings suggest that mPR and classical Pgr could potentially interact within the same neurons to mediate P_4 effects. Although E_2 has also been shown to regulate mPRα and mPRβ subtypes in the human myometrium (44), its regulation of mPR in the brain has only recently been examined. Using quantitative PCR, Intlekofer and Petersen (45) reported increases in mPRβ, but not mPRα, mRNA after E_2 treatment in the anteroventral periventricular nucleus and sexually dimorphic nucleus of the POA, two sexually dimorphic brain areas thought to be involved in controlling reproductive neuroendocrine function and behaviors. Two other potential targets for rapid P_4 actions [PR membrane components (Pgrmc)-1 and -2] are up-regulated by combined treatment with E_2 and P_4, whereas treatment with E_2 alone has no effect (45). Such findings indicate that mPRβ may be the more estrogen-responsive mPR in the CNS of rodents. The spatial and temporal correlations of mPR and classical Pgr in the hypothalamus and POA around the time of behavioral estrus prompted our current investigations into the expression and regulation of mPR in these regions.

Materials and Methods

Reagents and chemicals

All steroids and protease inhibitor I cocktails were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). Antibodies used in the study have been specified where appropriate. The reagents for electrophoresis and Western immunoblotting were purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals were of reagent grade and purchased from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

Animals and procedures

Female Sprague Dawley rats (~250 g) were obtained from Charles River Laboratories (Wilmington, MA) and housed at the Arizona State University Department of Animal Care and Technologies with food and water available *ad libitum*. For mRNA analysis, animals underwent bilateral ovariectomy under isoflurane anesthesia and 2 wk later were killed by decapitation. Brains were removed and immediately frozen on dry ice and stored at −80 C. For immunohistochemistry, binding assays and Western immunoblot analyses, ovariectomized Sprague Dawley rats (180–200 g) were obtained from Charles River Laboratories within a week after surgery. The animals were housed in the vivarium at Baylor College of Medicine, maintained on a 12-h light, 12-h dark cycle (lights on at 0600 h), and given food and water *ad libitum*. Four to six weeks after their arrival, the rats received sc injections of 2 μg E_2 benzoate/100 μl safflower oil or

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safflower oil alone and were killed 48 h after treatment. This time course has been shown to reliably increase classical Pgr in the hypothalamus and the POA of the rat brain (4, 6, 41, 42). All animals were killed under anesthesia (combination of ketamine 42.8 mg/ml, xylazine 8.6 mg/ml, and acepromazine 1.4 mg/ml). For immunohistochemical studies, anesthetized rats were intracardially perfused with 50 ml 0.9% saline followed by 200 ml 4% paraformaldehyde. Brains were removed from the skull and placed in the same fixative at 4 C overnight. The next morning, brains were transferred into a 30% sucrose cryoprotection solution, where they remained at 4 C until sectioning.

For Western blot analyses and binding assays, the brains were isolated from the cranial cavity and placed in cold artificial cerebrospinal fluid. Fresh brain dissections were carried out at 4 C. Using a McIlwain tissue chopper, the POA slab was cut through the middle of the optic chiasm as the caudal boundary. A rostral cut was made 1 mm anterior. The anterior commissure was used as a landmark to demarcate the superior border of the POA. The second cut was bounded rostrally by the caudal edge of the optic chiasm and caudally by the caudal edge of the mammillary bodies. The slabs were placed on a cold microscope stage, and areas of interest were viewed under a dissecting microscope with transillumination (Zeiss Stereo Discovery V8). Bilateral punches were made using the Palkovits punch method to dissect the hypothalamus and POA using a 1-mm internal diameter stainless steel punch, following the atlas of Paxinos and Watson (46). The cerebral cortex included the frontal cortex without the white matter. The punches were immediately frozen on dry ice/isopropanol and stored at −80 C until further analyses. All animal studies were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committees of Baylor College of Medicine and Arizona State University and were in compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Membrane PR binding assay

Binding of [1,2,6,7,^{3}H]P_4 ([^{3}H]P_4, 100Ci/mmol; PerkinElmer, Waltham, MA) to plasma membranes prepared from rat cortex, hypothalamus, and POA was conducted as described previously (47) with minor modifications. Membrane pellets for each brain region were prepared by pooling tissues, per region, from six animals. Plasma membrane pellets reconstituted in ice-cold HAED buffer [25 mM HEPES, 10 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol (pH 7.6). 0.5 mg protein/ml] were added to one set of tubes containing [^{3}H]P_4 alone (total binding) and another set of tubes containing [^{3}H]P_4 and 100-fold excess nonradiolabeled P_4 (nonspecific binding) and incubated at 4 C for 30 min. For saturation analysis [^{3}H]P_4 over the range of 0.5–8.0 nM was incubated with the membrane fractions. Saturation and Scatchard analyses were conducted by nonlinear regression using the Prism GraphPad program (GraphPad Software, San Diego, CA). Affinity (K_d) and capacity (B_{max}) of [^{3}H]P_4 binding was calculated from nonlinear curve fitting. Two-point competition assays were conducted with P_4, the selective mPR agonist 10-ethenyl-19-norprogesterone (Org OD 02-0; N.V. Organon, Oss, The Netherlands) and the nuclear Pgr agonist R5020 (PerkinElmer) at two concentrations, 10^{-7} and 10^{-6} M. The competitors were incubated with 2 nM [^{3}H]P_4, and plasma membrane preparations and the displacement of [^{3}H]P_4 binding by the competitors was expressed as a percentage of the maximum specific binding of [^{3}H]P_4. [^{3}H]P_4 bound to the plasma membranes was separated from free by rapid filtration through GF/B glass fiber filters (Whatman, Clifton, NJ) presoaked in assay buffer using a Brandel Semi Auto Harvester (Gaithersburg, MD). The filters were washed twice with 12.5 ml assay buffer, and the bound radioactivity was measured by scintillation counting.

Brain microdissection, RNA isolation, and quantitative real-time PCR

Frozen brains from ovariectomized female rats were sectioned at 300 μm using a Leica CM3050S cryostat (Leica, Buffalo Grove, IL). Ovariectomized rats were used to obtain an invariable hormonal milieu for comparison of mPR levels across regions and were not intended for assessment of estrogen regulation. The cortex, POA, and hypothalamus were identified using a brain atlas (46), and areas were punched bilaterally using a 1-mm diameter stainless steel cannula. Tissue was homogenized in guanidium isothiocyanate supplemented with β-mercaptoethanol, and RNA was extracted using a standard phenol/chloroform/isoamyl procedure (48). Purity and concentration of RNA was confirmed spectrophotometrically using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE), and 1 μg total RNA per sample was reverse-transcribed using the iScript cDNA synthesis kit (Bio-Rad). The resulting cDNA was quantified using a fluorescent detection reagent (Molecular Probes, Eugene, OR). The quantity of cDNA in each PCR was normalized based on the fluorescent quantification, and real-time RT-PCR was performed using a Roche LightCycler 480 employing SYBR green detection chemistry. The initial template for each gene was quantified by comparison with a standard curve generated with product formed by the respective primers. The following mPR-specific primers were used: mPRα (accession NM_001034081; sense 5′-GTGCACCGCATCATAGTGTC-3′, antisense 5′-TGATAGTCCAGCGTCACAGC-3′) and mPRβ (accession NM_001014099; sense 5′-CTGCAGCCTCT-TGGCCCACC-3′, antisense, 5′-CAGCCGCCGGCAGGAA-GAAA-3′). The real-time RT-PCR conditions were 95 C for 3 min, followed by 50 cycles of 95 C for 15 sec and 60 C for 1 min. After the last PCR cycle, each sample was subject to thermal melting curve analysis according to the Roche LightCycler 480 software protocol (Roche, Indianapolis, IN). For each RNA sample, a no-reverse-transcriptase reaction was run in parallel with cDNA synthesis and measured by RT-PCR to control for genomic DNA contamination. Each RT-PCR was verified for a single PCR product of expected size using thermal melting curve disassociation. Furthermore, PCR products of each primer were checked for correct size using gel (2%) electrophoresis. Absolute mRNA values were determined using standard curves constructed from isolated PCR product.

Western immunoblotting

A specific mPRβ polyclonal antibody was generated by a commercial vendor (Sigma-Genosys, Woodlands, TX), against a 15-amino-acid peptide sequence (KILE<u>D</u>GLPKMPCTVC) in the N-terminal region of human mPRβ, which differs at only at one position (*bold* and *underlined*) in the corresponding region of rat mPRβ, and used for Western blot analysis of rat brain extracts. This antibody has previously been validated for use with rodent tissues and cells, including the demonstration of decreased immunostaining of the protein band in Western blots of rat neuronal cell line extracts after treatment with mPRβ small inter-

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fering RNA (30, 32). Plasma membrane fractions were prepared from tissues by homogenization with a 2-ml hand-held glass homogenizer in HAED [25 mM HEPES, 10 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA (pH 7.6)] buffer containing protease inhibitors (Pierce, Rockford, IL). The homogenate was centrifuged at 1000 × *g* for 7 min to pellet the nuclei and cell debris, and the supernatant was the transferred to another tube and centrifuged at 20,000 × *g* for 20 min to pellet the crude plasma membrane fraction. The pellets containing the crude membrane fractions were resuspended in HAED buffer and layered on top of 0.5 M sucrose and centrifuged at 9500 × *g* for 45 min. The resulting partially purified membrane preparations were mixed with 5× reducing Western blot sample mix (Pierce) and incubated at room temperature for 20 min. The solubilized membrane proteins (~15 μg/lane) were loaded on two separate 10% polyacrylamide gel and separated by SDS-PAGE (49). The separated proteins were transferred to nitrocellulose membranes (Bio-Rad) according to the method of Towbin *et al.* (50). The membranes were washed with Tris-buffered saline (TBS) buffer [50 mM Tris, 100 mM, NaCl (pH 7.4)] followed by an hour blocking in 5% nonfat milk in TBS containing 0.1% Tween 20. The membranes were incubated overnight at 4 C with the mPRβ antisera (1:2500 dilution) and washed with PBS [137 mM NaCl, 10 mM phosphate, 2.7 mM KCl (pH of 7.4)]. Antibody binding was revealed by incubation with donkey antirabbit horseradish peroxidase-conjugated IgG (1:10,000 dilution; Cell Signaling Technology, Boston, MA) followed by chemiluminescence detection with the enhanced chemiluminescence reagent (Pierce) and exposed to x-ray film to visualize the specific protein bands. Membranes from untransfected human MDA-MB-231 cells (mPRβ positive) and cells stably transfected with human mPRβ were used as positive controls. Further validation of mPRβ antibody was also performed by Western blot analysis of membrane preparations from MB-MDA-231 cells stably transfected with mPRα, mPRβ, mPRγ, mPrδ, and mPRϵ, probed with the specific mPRβ antibody.

Tissue processing and mPRβ immunohistochemistry

Paraformaldehyde-fixed brains were sectioned into three series at 35 μm thickness using a Leica CM3050S cryostat (Leica Microsystems GmbH, Wetzlar, Germany), and tissue was processed for immunocytochemical detection of mPRβ and Pgr using a free-floating sections. Briefly, the immunocytochemical method consisted of incubating free-floating tissue sections for 10 min in 1% hydrogen peroxide in TBS to block endogenous peroxidase. Tissue was next rinsed in TBS, incubated in 4% normal goat serum (NGS) in TBS, followed by overnight incubation with polyclonal antibodies to mPRβ (1:5000 dilution) or Pgr (1:500 dilution) in 4% NGS in TBS. The next day, tissue was washed in TBS, incubated for 1 h in goat antirabbit biotinylated IgG in 4% NGS in TBS, again rinsed in TBS, and incubated for 1 h in avidin-biotin complex in TBS. After rinses in TBS, tissue was developed for 10 min to visualize mPRβ- or Pgr-positive cells using diaminobenzidine as the chromogen. Tissue was then mounted on glass slides, and sections were air dried overnight at room temperature. The next day, slides were processed through increasing alcohols, cleared with xylene, and coverslipped with Permount (Fisher Scientific). The specificity of the mPRβ antibody was tested and confirmed by preadsorption of the antibody with excess peptide antigens (0.05 mg peptide/1 ml antibody). Preadsorption completely prevented the immunoreactive signal (Fig. 3H). Antibodies to mPRβ used in the study were the same as those used for Western immunoblotting described above. Classical Pgr antibodies were obtained from Dako [Carpinteria, CA; rabbit antihuman PR A0098 lot 135(011)].

Microscopic analysis of mPRβ distribution

Analysis of mPRβ distribution and density was conducted on a Zeiss Axioskop light microscope equipped with a MicroBright-Field Bioscience CX9000 video camera (MicroBrightField Inc., Williston, VT). Brain regions were identified using the rat brain atlas of Paxinos and Watson (46). Three independent investigators determined immunoreactive-cell density and intensity by assigning ratings based on the density and intensity of immunoreactive product within selected brain regions of three rats. Values were applied on a scale ranging from −, representing no immunoreactivity, to ****, designating the greatest density/intensity of immunoreactive cells found in the brain. For comparison of E_2 effects, mPRβ-immunoreactive (ir) cells were counted bilaterally in two consecutive sections (105 μm apart) within selected brain regions, which showed distinct and quantifiable cell labeling; n = 5 animals per treatment (E_2 benzoate and vehicle). Cells were counted within a fixed grid size for each area examined, with data expressed as counts per area. Counts per section were averaged to obtain one data point per animal. Pgr-ir cells were quantified using the same methodology within the medial POA, and all counts were performed using Neurolucida version 7 software (MicroBrightField).

Results

P_4 binding to rat brain plasma membranes

As a prelude to examining the role of E_2 regulation of mPR, we examined membrane preparations from the hypothalamus, POA, and cortex for their ability to bind P_4. Specific [^{3}H]P_4 binding was detected on plasma membranes prepared from the three rat brain regions. Saturation binding and Scatchard analyses identified a single, high-affinity (K_d), low-capacity (B_{max}) progestin binding site on plasma membranes from the cortex (K_d = 5.42 ± 0.38 nM, B_{max} = 0.055 ± 0.0004 nM), hypothalamus (K_d = =9.88 ± 1.10 nM, B_{max} = 0.095 ± 0.024 nM) and POA (K_d = 10.1 ± 0.87 nM, B_{max} = 0.076 ± 0.004 nM) (Fig. 1; A, C, and E). Competition studies showed that [^{3}H]P_4 binding was displaceable, with 10^{-7} and 10^{-6} M (100 nM and 1 μM) nonradiolabeled P_4 displacing more than 80% of the [^{3}H]P_4 binding to the membrane fractions (Fig. 1, B, D, and F). The selective mPR agonist Organon OD 02-0 (51) was also an effective competitor of [^{3}H]P_4 binding to the rat brain membrane preparations, with a binding affinity slightly lower than that of P_4. In contrast, the synthetic nuclear Pgr agonist, R5020, which has low binding affinity for human mPR (47), did not show any binding of the rat brain mPR (Fig. 1, B, D, and F).

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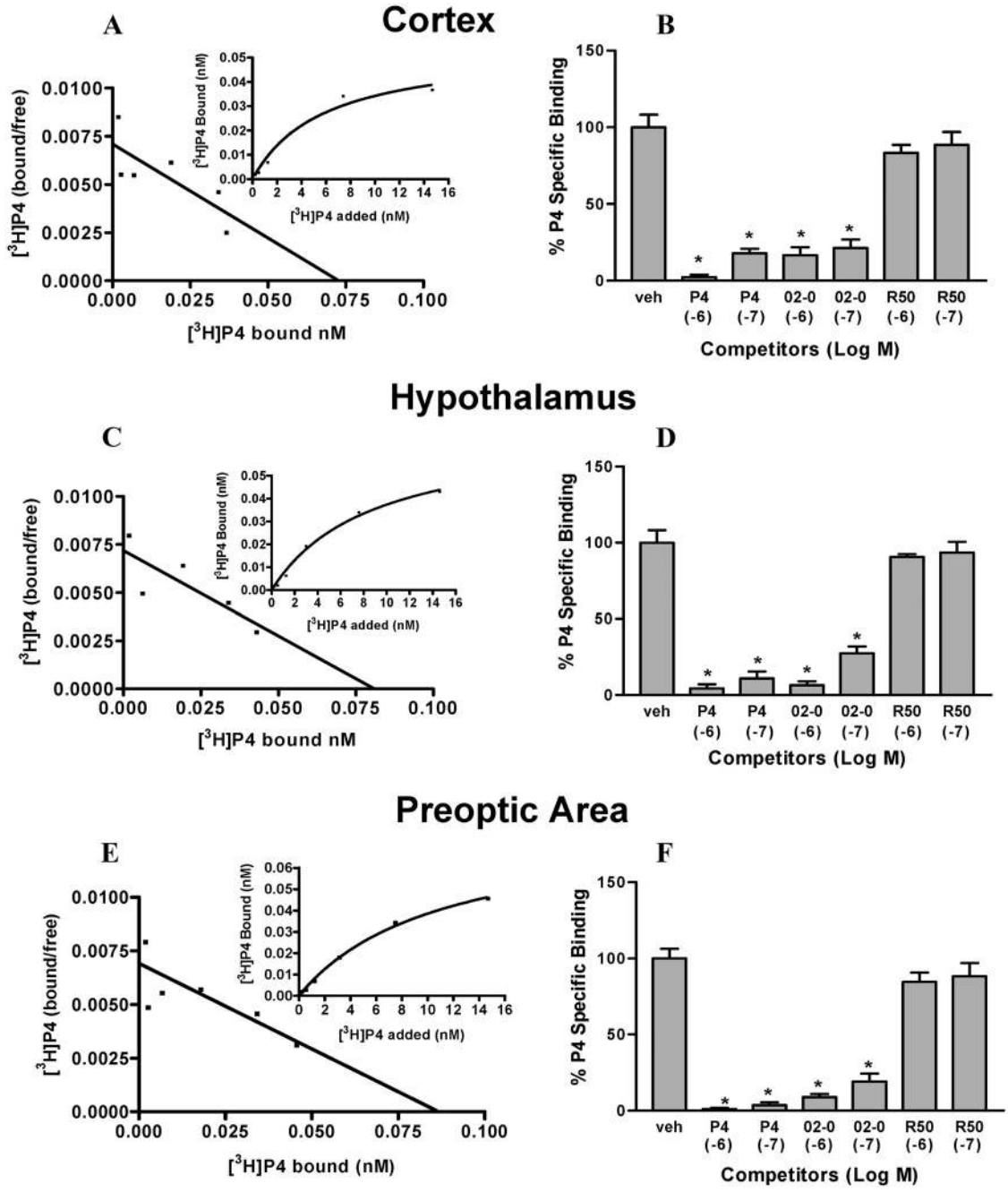

FIG. 1. P_4 binding to plasma membranes of rat brain cortex, hypothalamus, and POA. A, C, and E, Representative saturation curves and Scatchard plots of specific [^{3}H]P_4 binding analyses showing single, high-affinity (K_d), low-capacity (B_{max}) progestin-binding sites on plasma membranes from the cortex (A) (K_d = 5.42 ± 0.38 nM, B_{max} = 0.055 ± 0.0004 nM), hypothalamus (C) (K_d = 9.88 ± 1.10 nM, B_{max} = 0.095 ± 0.024 nM) and POA (E) (K_d = 10.1 ± 0.87 nM, B_{max} = 0.076 ± 0.004 nM); B, D, and F, two-point competition assays of P_4, Organon OD 02-0 (02-0), and R5020 (R50) binding to membranes from the cortex (B), hypothalamus (D), and POA (F). *, Significantly different from vehicle controls ($P < 0.001$, Dunnett's multiple-comparison test); n = 6.

mPRα and mPRβ mRNA analysis

Because the membrane-binding assay does not discern the subtype of mPR present in membranes, we used quantitative RT-PCR analysis to determine levels of mPR mRNA in the cortex, POA, and hypothalamus. Quantitative RT-PCR showed higher levels of mPRβ than mPRα (Fig. 2A) in all brain areas examined. mPR mRNA levels varied somewhat by region, and levels for both mPRα and mPRβ tended to be greatest in the cortex (Fig. 2A).

Western blot analysis

Because the cortex showed highest levels of mPRβ mRNA, Western blot analysis was performed on cortical membranes to verify the specificity of the mPRβ antibody.

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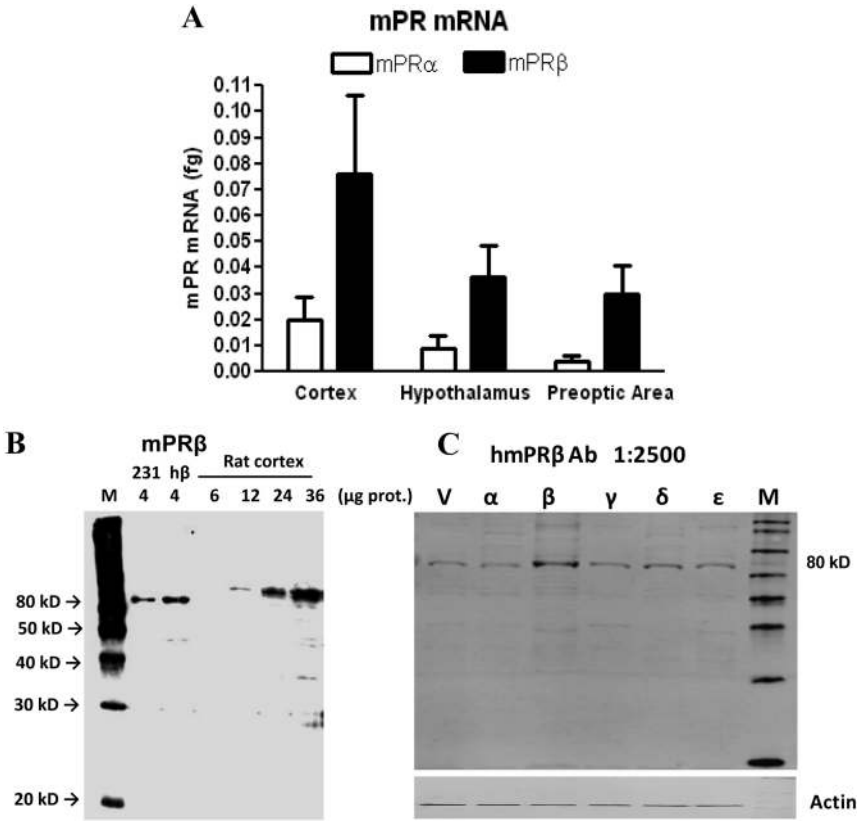

FIG. 2. mRNA analysis of mPRα and mPRβ and Western immunoblotting for mPRβ. A, mPRα and mPRβ mRNA levels from the cortex, hypothalamus, and POA as determined by quantitative RT-PCR; n = 5 per group for mRNA analysis. B, Western blot analysis shows predominantly 80-kDa bands for the mPRβ antibody in brain membrane extracts. C, Western blot analysis of MB-MDA-231 cells stably transfected with mPR with the specific mPRβ antibody showing the presence of a strong band in the membrane fraction prepared from cells expressing mPRβ and actin loading controls. 231, MDA-MB-231 cells transfected with vector alone; hβ, overexpression with mPRβ; V, vector; α, mPRα; β, mPRβ; γ, mPRγ; δ, mPRδ; ϵ, mPRϵ; μg prot., membrane protein loaded in each lane; M, molecular weight marker.

This showed the presence of 80-kDa immunoreactive bands, which likely represent mPRβ dimers (Fig. 2B). Examination of the specificity of the mPRβ antibody in MDA-MB-231 cells stably transfected with mPRα, mPRβ, mPRγ, mPrδ, and mPRϵ or vector alone showed stronger staining of the 80-kDA band in plasma membrane preparations from mPRβ-transfected cells compared with those transfected with vector alone or other mPR (Fig. 2C), further indicating the specificity of the immunoreactions. The expression of mPRβ in MDA-MB-231 cells was confirmed by RT-PCR (Supplemental Fig. 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org).

mPRβ immunohistochemical analysis

The mPRβ antibody was next used to examine the distribution of mPRβ in the rat brain. Immunohistochemistry showed that mPRβ was distributed throughout the brain (Table 1 and Figs. 3 and 4) with generally high levels in many regions of the thalamus (Fig. 3, B and F) and cortex (Figs. 3G and 4, D–F), although both regions also included areas devoid of labeling (*e.g.* central medial thalamic nucleus and infralimbic cortex). The hypothalamus also showed a high density of mPRβ-ir neurons within specific nuclei including the paraventricular nucleus (Fig. 3J), supraoptic nucleus (Fig 3, D and H), and ventromedial nucleus. Basal forebrain regions (horizontal diagonal band, vertical diagonal band, and medial septum; Fig. 3I) also displayed extensive labeling. In the hindbrain, nuclei including the oculomotor nucleus, red nucleus (Fig. 3K), substantia nigra, and ventral tegmental area showed greatest expression. Areas of the hippocampus displayed variable labeling intensity with the CA2 and CA3 regions showing moderate to high levels and the CA1 and dentate gyrus divisions showing weak immunoreactivity (Fig. 4, A–C). The amygdala (Fig. 3, A and E) showed consistently low levels of mPRβ-ir. In general, cells showed strong punctate mPRβ labeling in the membrane with some cells also showing a weaker distributed pattern in the cytoplasm (see Fig. 3, A–D). The mPRβ label was also present in some axons, most prominently within the middle cortical layers (Fig. 4F). This cellular distribution of mPRβ protein labeling is analogous to the pattern described in cells of the mouse spinal cord (33). Specificity of mPRβ antibody was confirmed by preadsorption with the antigenic peptide, which eliminated immunoreactive labeling in the brain (Fig. 3, K and L).

Estrogen regulation of mPRβ expression

Student's *t* tests revealed a significant increase in mPRβ-ir cells after E_2 treatment within the medial septum [$t(8) = 2.68$; $P < 0.05$; Fig. 5D) but not in the oculomotor nucleus, horizontal diagonal band, or medial POA ($P > 0.05$; Fig. 5, A–C). As a control, and confirming previous reports, classical Pgr-ir was increased in the medial POA after E_2 treatment ($P < 0.001$; Supplemental Fig. 2) (37, 38).

Discussion

In this study, we demonstrate the presence of high-affinity, specific P_4 binding, characteristic of mPR using saturation

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TABLE 1. Distribution of mPRβ-ir cells in the adult female rat brain

Region	mPRβ density of labeled cells	mPRβ intensity of label
Telencephalon		
Frontal cortex	**	**
Insular cortex	*	**
Piriform cortex	**	**
Parietal cortex	***	**
Ventral pallidum	–	–
Orbital cortex	*	*
Cingulate cortex	*	*
Infralimbic cortex	–	–
Dorsal peduncular cortex	–	–
Dorsal tenia tecta	*	*
Medial septum	***	***
Lateral septum	*	*
Islands of Calleja	**	**
Bed nucleus of the stria terminalis, medial	–	–
Basomedial amygdaloid division	*	*
Central amygdaloid nucleus	–	–
Medial amygdaloid nucleus	*	*
Cortical amygdaloid nucleus	*	*
Basolateral amygdaloid nucleus	*	*
Hippocampus		
CA1	*	**
CA2	**	**
CA3	**	***
Dentate gyrus	*	*
Epithalamus		
Medial habenular nucleus	–	–
Lateral habenular nucleus	*	*
Thalamus		
Centrolateral thalamic nucleus	*	*
Central medial thalamic nucleus	–	–
Paracentral thalamic nucleus	–	–
Ventromedial thalamic nucleus	**	***
Reticular thalamic nucleus	***	**
Ventral posterolateral thalamic nucleus	*	*
Ventral posteromedial thalamic nucleus	**	***
Posterior thalamic nuclear group	*	*
Zona incerta	*	*
Hypothalamus		
Horizontal diagonal band	***	***
Organum vasculosum of lamina terminalis	*	**
Suprachiasmatic nucleus	*	*
Medial preoptic area	**	**
Periventricular hypothalamic nucleus	*	*
Paraventricular nucleus, magnocellular	****	***
Paraventricular nucleus, parvocellular	**	***
Supraoptic nucleus	****	****
Lateral hypothalamic area	**	*
Arcuate nucleus	**	*
Ventromedial hypothalamic nucleus	***	**
Mesencephalon		
Substantia nigra–reticular	**	**
Substantia nigra–lateral	**	**
Deep mesencephalic nucleus	**	**
Oculomotor nucleus	****	****
Red nucleus	****	****
Interpeduncular nucleus	–	–
Rostral linear nucleus of the raphe	*	*
Ventral Tegmental Area	***	***
Pons		
Mesencephalic trigeminal nucleus	**	***
Dorsal nucleus of the raphe	*	*
Central gray	–	–
Trochlear nucleus	**	**

Intensity of immunoreactive signal was scored by three investigators using the following scale: –, absence of label; *, low mPRβ density/intensity of label; **, medium mPRβ density/intensity of label; ***, high density/intensity of label; ****, greatest density/intensity of label found in the brain. The average of the three scores is presented in this table.

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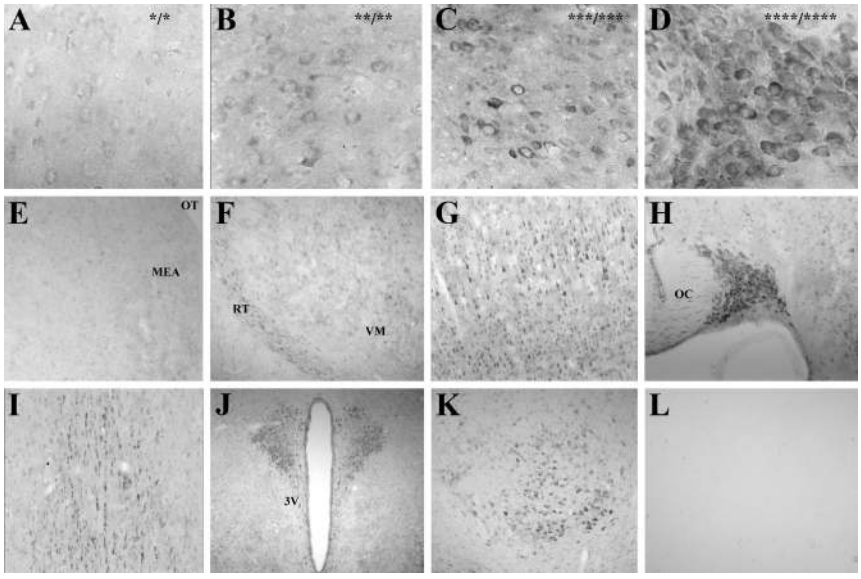

FIG. 3. Representative images of mPRβ-ir in selected brain regions of the female rat brain. A–D, High-magnification images of mPRβ-ir cells in the medial amygdala (A), ventromedial thalamic nucleus (B), parietal cortex (C), and supraoptic nucleus (D) showing varying levels of labeling intensity. *Asterisks* provide indication of rating scale used to determine relative levels of mPRβ density/intensity as shown in Table 1. E–L, Low-magnification images of amygdala (E), thalamus (F), cortex (G), supraoptic nucleus (H), medial septum (I), paraventricular nucleus (J), red nucleus (K), and red nucleus after preadsorption with mPRβ peptide antigens (L). Images indicate weakly labeled cells in the amygdala, moderate labeling intensity within the thalamus, and high levels of mPRβ-ir in the cerebral cortex particularly in the middle/inner neuronal layers. The supraoptic nucleus showed dense and strongly labeled mPRβ-ir cells. Tissue sections were 35 μm thick. MEA, Medial amygdala; OC, optic chiasm; OT, optic tract; RT, reticular thalamic nucleus; 3V, third ventricle; VM, ventromedial thalamic nucleus.

binding and Scatchard analyses of plasma membranes prepared from rat cortex, hypothalamus, and POA. We further show the regional distributions of both mPRα and mPRβ mRNA in the ovariectomized female rat brain using quantitative real-time PCR and found that overall expression level of mPRβ was higher than that of mPRα. We therefore focused on mPRβ and investigated the regional distribution of mPRβ protein in the female rat brain using immunohistochemistry. mPRβ was widely distributed throughout the female rat brain with consistently high levels in select hypothalamic, forebrain, hippocampal, and midbrain regions as well as throughout the thalamus and cortex. We also investigated whether mPRβ-ir was responsive to E_2 treatment. E_2 treatment caused an increase in mPRβ-ir cell number in the medial septum but not in the medial POA, horizontal diagonal band, and oculomotor nucleus in the rat brain.

The characteristics of [^{3}H]P_4 binding to plasma membranes showed high affinity, limited capacity, and displaceable [^{3}H]P_4 binding, which is typical of membrane progestin receptors. The progestin competition studies suggest [^{3}H]P_4 is not binding to the nuclear Pgr on the rat brain membranes because the high-affinity nuclear Pgr ligand R5020 did not compete for [^{3}H]P_4 binding at concentrations up to 1 μM. On the other hand, the steroid-binding profile of the membrane preparations, with high binding affinities for P_4 and Organon OD 02-0 and a low affinity for R5020, are characteristics of mPR (47, 51). Dissociation constants are in the same range (K_d 5–10 nM) as those previously reported for mPR (30, 47). Receptor concentrations in these brain regions (B_{max} 0.55–0.95 nM) are also similar to those found in other vertebrate tissues and cells (30, 52, 53). Together, these results indicate that the P_4 binding to rat cortex, hypothalamus, and POA membranes is primarily mediated through mPR.

The mPRβ expression levels were greater than mPRα levels in the female rat brain. This result indicates that mPR expression in the rat may be similar to that of the human where mPRβ is the primary mPR subtype expressed in the

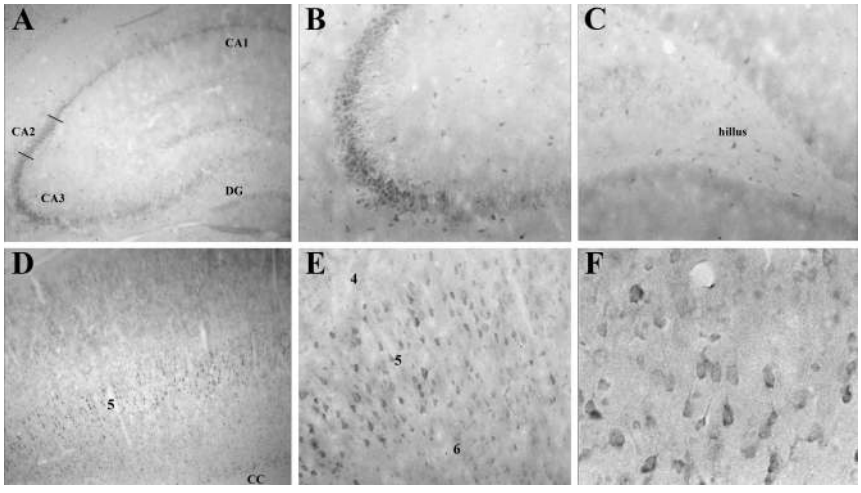

FIG. 4. Distribution of mPRβ-ir within the hippocampus and cortex. A and B, Low-magnification image of the hippocampus (A) and higher-magnification image of the CA3 region (B), which contained many mPRβ-ir cells; C, mPRβ-ir cells were sparse in the dentate gyrus with some labeled cells found in the hillus region; D–F, low-magnification image of the cerebral cortex (D) and higher-magnification images of inner cortical layers (E and F), which contained dense and strongly labeled mPRβ-ir-positive cells, particularly within layer 5 (F). Many mPRβ-expressing cells in layer 5 displayed morphological characteristics of pyramidal cells. Layer numbers (4–6) are indicated. CC, Corpus callosum.

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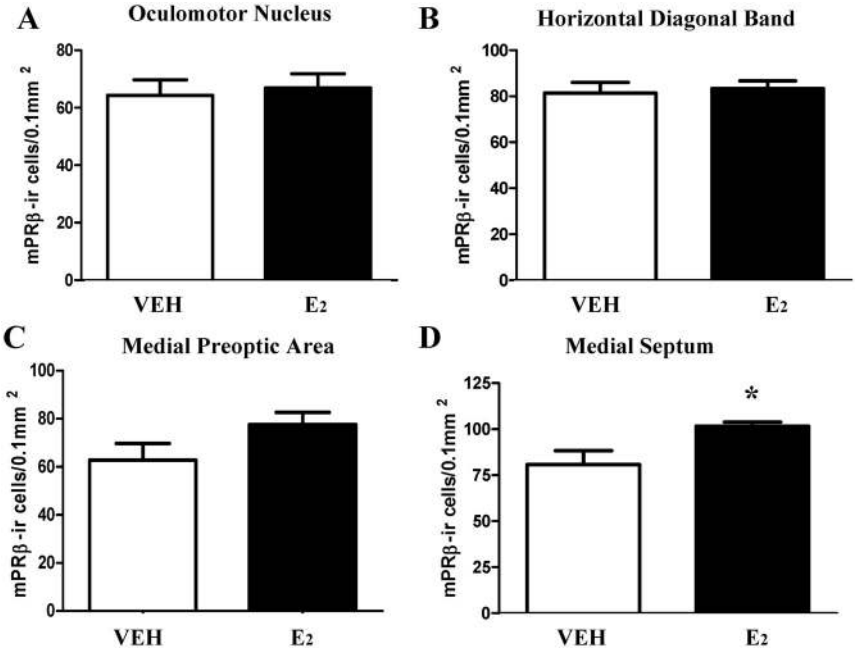

FIG. 5. Estrogen treatment alters mPRβ expression in a brain region-selective pattern. mPRβ-ir cells were counted in brain regions of ovariectomized female rats killed 48 h after treatment with E_2 benzoate. A–C, The number of mPRβ-ir cells did not significantly differ between E_2-treated and vehicle-treated rats in the oculomotor nucleus (A), horizontal diagonal band (B), or medial POA (C); D, a significant increase in mPRβ expression was found in the medial septum after E_2 treatment; n = 5 per treatment. *, $P < 0.05$.

CNS (26). Comparisons between brain regions (cortex, POA, and hypothalamus) showed greatest levels of mPRβ in the cortex, which is consistent with regional differences in mPRβ-ir that we describe in tissue sections.

Immunohistochemical analysis of mPRβ in the female rat brain revealed high levels of expression in a variety of thalamic nuclei examined as well as moderate levels within the cortex. Specific nuclei within the hypothalamus expressed high levels of mPRβ including the paraventricular and arcuate nuclei. Basal forebrain regions including the medial septum and horizontal and vertical diagonal band also exhibited extensive expression of mPRβ, as did the oculomotor nucleus, red nucleus, ventral tegmental area, and substantia nigra of the midbrain. In contrast, mPRβ protein levels were generally lower in all subregions of the amygdala and in the hippocampal CA1 and dentate gyrus regions, although populations of cells within the CA2 and CA3 divisions showed dense mPRβ-ir. The broad, but regionally selective, distribution of the mPRβ suggests that it may regulate a wide range of functions including cognitive, sensory, motor, behavioral (sexual, aggression, and emotional), and neuroendocrine. In contrast, the consistently lower expression of mPRβ in the amygdala indicates mPRβ plays a minimal role in the regulation of emotional and fear-related memory functions associated with this region. Although mPRβ protein expression was found at high levels in several hypothalamic nuclei known to be regulated by P_4, it was also highly expressed in cortical, thalamic, and midbrain regions (*e.g.* red and oculomotor nuclei) where less is known regarding P_4 actions. P_4 has been demonstrated to be neuroprotective after brain injury in both the cortex and thalamus (54, 55); therefore, it is possible that neuroprotection may be mediated in part via activation of mPRβ. P_4 has also been demonstrated to bind cells in thalamic nuclei (56) that are void of Pgr (57), although effects on functions associated with the thalamus are unknown. P_4 regulation of midbrain regions, specifically the red nucleus and oculomotor nucleus, which contain extensive mPRβ-ir, is currently unknown.

The distribution and level of mPRβ protein presented here is largely consistent with a recent report showing the distribution of mPRβ mRNA in the rodent brain using *in situ* hybridization (34). One exception is that we report expression in the hypothalamus with regional localization: specific nuclei showing a high expression and others showing little to no expression as opposed to Intelkofer and Peterson (34), who report consistently low hypothalamic mPRβ levels. This may indicate a nonlinear relationship between transcript and protein levels unique to certain hypothalamic nuclei. The majority of cells expressing mPRβ appeared to show neuronal characteristics; however, studies using dual-label immunohistochemistry would be necessary to confirm the phenotype of these cells. It is also possible that mPRβ is present in glial cells within the rat brain, although this contrasts with the findings from mouse spinal cord that indicated that glial cells do not express mPRβ and that expression is largely confined to neurons (33).

Treatment with E_2 increased mPRβ-ir in the medial septum but did not alter mPRβ expression levels in the medial POA, horizontal diagonal band, and oculomotor nucleus. The lack of effect of E_2 treatment in the oculomotor nucleus is consistent with the minimal expression of ERα and ERβ (58). The horizontal diagonal band shows high levels of mPRβ and moderate levels of ERα and ERβ (59); however, E_2 failed to elicit effects on mPR-ir within this region. Similarly, the medial POA contains very high levels of both ER subtypes (60), but E_2 also failed to significantly increase mPRβ-ir, although there was a trend toward elevated expression. This finding is in contrast to a recent report where mPRβ mRNA levels, obtained by

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quantitative PCR, were shown to increase after E_2 treatment (45). This discrepancy could be due to the differences in the specific regions within the medial POA that were analyzed, inconsistencies between E_2 effects on mRNA and protein levels, or subtle differences in E_2 treatment regimens. In the present study, rats were administered E_2 benzoate by a single injection 48 h before being killed (a regimen followed to mimic E_2 changes across the estrous cycle), whereas Intlekofer and Petersen (45) implanted E_2-containing capsules for a longer, 56-h exposure, before the animals were killed. Nonetheless, our data indicate that the timing of our estrogen treatment was sufficient to alter expression of the classical Pgr (Supplemental Fig. 2). However, it is possible that the timing of estrogen-induced alterations in mPRβ expression may differ from Pgr. Elevated mRNA levels in this region also may not necessarily translate to an increase in the number of cells expressing the protein but, instead, the amount of receptor per cell. Moreover, it is possible that protein levels of mPRβ within individual cells may have been decreased in the medial POA, a difference that we could not detect in our analysis, although additional studies are needed to test this. Other regions showing high levels of ER, such as the ventromedial hypothalamus (which expresses very high levels of ERα) may increase mPRβ after E_2 treatment; however, cell counts were not performed within this region due to extensive cellular overlap that made accurate quantification unattainable. Studies are in progress to examine E_2 regulation of mPR binding across brain regions based on our findings of region-specific regulation of mPRβ expression.

Because the medial septum was the only region to show a significant increase in mPRβ mRNA expression after E_2 treatment, this indicates that cells within the medial septum are particularly sensitive to estrogen effects on mPRβ expression. Both ER subtypes are present within the medial septum (60, 61), indicating a mechanism through which E_2 can alter mPRβ expression, although additional studies using ERα- and ERβ-selective ligands are needed to explore individual contributions of ER in mediating these effects. The medial septum contains a large number of cholinergic cells (62), and treatment with E_2 increases cholinergic activity in this region (63). Cholinergic cells within the medial septum also show a high colocalization with ERα, which is greater compared with other cholinergic regions of the basal forebrain (59, 62). It is therefore possible that this phenotype of neurons within the medial septum is particularly responsive to E_2 and may represent a large population of cells that express mPRβ, although additional immunohistochemical studies are needed to test this hypothesis. The medial septum has been implicated in the regulation of aggression, anxiety, and memory-related functions (64–66). Therefore, our current finding suggests that estrogen may modulate P_4 regulation of these functions by increasing the availability mPRβ. P_4 has been demonstrated to influence each of these behaviors (67–69); however, the specific receptors involved in mediating these actions are less clear. Previous studies indicate that other membrane-associated Pgr subtypes including Pgrmc1 and Pgrmc2 may also show alterations in protein after E_2 treatment (45) and are therefore another potential mechanism through which E_2 can regulate rapid P_4 actions. Additional studies are needed to test E_2 effects on the regulation of these proteins.

In summary, our findings indicate that mPRβ may be the primary mPR subtype in the brain of female rats given the greater mRNA expression of mPRβ than mPRα. Immunohistochemical examination of mPRβ indicates a broad distribution in the brain, implicating mPRβ in the regulation of a wide range of functions including sensory, motor, cognitive, behavioral, and neuroendocrine. Furthermore, E_2 regulation of mPRβ in the medial septum indicates a mechanism through which estrogens can regulate P_4 function within discrete brain regions to potentially impact behavior.

Acknowledgments

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