Non-nuclear Estrogen Receptor β and α in the Hippocampus of Male and Female Rats

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ABSTRACT: Estrogens play important roles in the brain, acting through two receptor types, ERα and ERβ, both recognized as transcription factors. In this study, we investigated the ERβ mRNA and protein expression in the male and female rat brain, focusing on the hippocampus, and comparing with well-known ERα expression patterns. Extranuclear ERβ localization, as shown by light microscopic immunocytochemistry and tissue fractionation experiments, was noted in the hippocampus, whereas nuclear ERβ was present in the amygdala. Despite these marked differences in subcellular localizations, similar expression levels of ERβ proteins as well as the profile of ERβ mRNA isoforms were observed in the two brain structures. ERα was localized to the nucleus more so than ERβ, yet not without an extranuclear component. Our results suggest that cytoplasmic estrogen receptors may play an important role in hippocampal physiology. © **2005 Wiley-Liss, Inc.**

KEY WORDS: estrogen receptor β; extranuclear localization; subcellular distribution; plasticity

INTRODUCTION

Estrogens play an important role not only in brain development, but also during its life span. Most studies on estrogens in the CNS have implicated them in the promotion of neuronal survival, growth, protection, and synaptogenesis (Chen et al., 1998; Ferrera and Caceres, 1991; Dubal et al., 1998; Woolley et al., 1990; Weiland, 1992; Woolley and McEwen, 1992, 1994), in the enhancement of basal synaptic responses (Ito et al., 1999; Farr et al., 2000), and of higher cognitive functions (Simpkins et al., 1997).

Two types of estrogen receptors, ERα and ERβ, operating as transcription factors, are the major mediators of the biological functions of estrogen (Jensen and Jacobson, 1962; Gorski et al., 1968; Evans, 1988). Both proteins are expressed in the brain in a variety of isoforms, studied mainly at the mRNA level (Lewandowski et al., 2002; Pfeffer et al., 1996). The mRNA for ERα is highly expressed in the areas of the brain that are responsible for reproduction, whereas ERβ mRNA is present mainly in the brain regions of nonreproductive function, such as the hippocampal formation and the amygdala (Shughrue et al., 1997; Osterlund et al., 1998). In gene knockout (KO) studies, ERα-deficient mice display impaired reproductive behaviors, while ERβ KO animals show intact reproduction but have morphological and functional abnormalities in the brain (Lubahn et al., 1993; Wang et al., 2001; Krezel et al., 2001). These results suggest that ERβ is a good candidate as the estrogen receptor participating in the regulation of nonreproductive brain functions.

In this report, we show the expression of the estrogen receptor mRNA and protein in the hippocampus of hormonally intact male and female rats. The proteins of ERα and ERβ differ, with respect to both their level of expression as well as their distribution within the hippocampal formation. In particular, an unexpected finding for transcription factor proteins is a strong extranuclear presence of ERβ.

MATERIALS AND METHODS

Animals and Their Treatment

Intact male and female Wistar rats weighing 200–250 g, from the Nencki Institute, were used. The rats were housed on a 12-h light/dark cycle ad libitum food and water. Animal experimentation reported in the present work has been conducted in accordance with the guidelines laid down by Polish regulations (Act on Animal Welfare) pertaining to the use of laboratory animals.

Immunocytochemistry

Primary antibodies

The following primary antibodies were used: ERα (rabbit: Upstate Biotechnology, Lake Placid, NY: 1:50,000, Santa Cruz. Biotechnology, Santa Cruz, CA MC-20, 1:10,000), ERβ (Z8P; rabbit: Zymed, 1:3,000), NeuN (mouse; Chemicon, Temecula, CA; 1:1,000), GFAP (mouse; Chemicon, 1:1,000), parvalbumin (mouse; Chemicon, 1:1,000), GAD65/67 (rabbit: Chemicon, 1:1,000), αCamKinaseII (αCamKII; mouse; Affinity Bioreagents, Golden, CO, 1:700).

Blocking peptides

Procedures. The following blocking peptides were used: blocking peptide to anti-ERα MC-20, mapping at the C-terminus of ERα of mouse origin (Santa Cruz Biotechnology); and peptide to block the anti-ERβ Z8P antibody, PA1-310B (Affinity Bioreagents), 19-amino acid sequence from amino acids 467–485 of ERβ.

The rats were anesthetized with pentobarbital and perfused with saline followed by 2% paraformaldehyde with 15% picric acid (Kritzer, 2002) in 0.1 M phosphate

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buffer, pH 7.4. The brains were stored in the same fixative for 24 h at 4°C and then cryoprotected in 30% sucrose solution, followed by gradual freezing on dry ice. Next, after equilibration to −20°C, the brains were cut on a cryostat (Cryocut 1800, Leica) in the coronal plane (35 μm, dorsal portion of rostral hippocampus, distance from bregma: −2.3 to −4.5). Immunocytochemistry on free-floating sections with polyclonal antibodies was carried out according to Shughrue and Merchenthaler (2001). Briefly, the sections were rinsed 3× for 5 min in cold phosphate-buffered saline (PBS) (0.1 M pH; Sigma, St. Louis, MO), followed with incubation in 0.1 M glycine. Following a 2 h 4°C blocking step, consisting of 10% normal goat serum (NGS), 1% bovine serum albumin (BSA), 1% H_2O_2 in phosphate-buffered saline (PBS) with 0.3% Triton X-100, the sections were incubated with primary antibody for 48 h at 4°C. Reactions were visualized with either 3,3′-diaminobenzidine (DAB staining kit, Vector, Burlingame, CA) or fluorochrome-conjugated secondary antibodies (Vector, 1:500). Tyramide-conjugated fluorochromes (TSA cyn3, Perkin-Elmer Life Sciences, 1:100) were used to visualize the ERα and ERβ proteins. For double-label immunocytochemistry, primary antibodies generated in different species (with the exception of anti-GAD65/67) were used. In the case of co-staining for GAD65/67 and the estrogen receptors, we have followed a commonly used protocol for sequential dual-labeling for same-species antibodies (Hart et al., 2001; Geurts et al., 2001; Uchihara et al., 2003), including controls to assess for possible antibody cross-reactivity. Briefly, incubation with either ERα or ERβ was carried out first, followed by secondary antibody incubation and signal visualization. Next, the sections were blocked once again and incubated with the second primary antibody (anti-GAD65/67). The rest of the procedure was the same as described above. The stained sections were examined under a light microscope (Olympus IX70), equipped with a color camera (Olympus DP50), as well as with a confocal laser microscope (Leica TCS SP2). The brains of at least five male rats were used for the immunocytochemistry studies.

Cell Fractionation

The hippocampi of three to four male Wistar rats were isolated and immediately frozen on dry ice. The protocol used was that adapted from Gray and Whittaker (1962). In brief, the tissue was thawed in homogenization buffer, consisting of Hepes 10 mM, EDTA 1 mM, sucrose 295 mM, dithiothreitol (DTT) 5 mM, pH 7.3, and homogenized on ice in a glass homogenizer. The tissues of the three animals were pooled. The homogenates were centrifuged for 10 min at 1,000*g* to isolate the nuclei, and the supernatant was subjected to a 55-min centrifugation at 17,000*g* to isolate the mitochondria. The supernatant from the second centrifugation was again centrifuged at 100,000*g* for 60 min, to obtain the endoplasmic reticulum (P3 pellet, suspended in homogenization buffer) and the cytosol (S). The collected fractions were treated with 5 × sodium dodecyl sulfate (SDS) solution (100 μM Tris-Cl, 4% SDS, 0.2% bromophenol blue dye, 20% glycerol, 200 μM dithreitol DTT), denatured at 100°C for 10 min, chilled on ice, and stored at −20°C, until used. Additional protein samples were collected before SDS treatment and used for calculation of concentration using the Bradford method. The protein fractions were used for immunoblotting, following the procedure outlined below. Each lane of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel contained 10 μg of protein.

Western Blot

For immunoblot analysis, the medial amygdala were collected from 30 animals, in contrast to hippocampal samples from single rats. The rat brain was cut into 500-μM-thick slices on a microtome. The medial amygdala was dissected out under a stereoscopic microscope. The position of the medial amygdala was determined according to the brain atlas of Paxinos and Watson (1998). Tissue from 30 animals was pooled and collected in TRI Reagent (Sigma). Total protein extract was isolated (TRI Reagent) and separated by SDS-PAGE. For immunochemical detection, the polyvinylidine difluoride (PVDF) membranes (Hybond, Amersham Biosciences, Piscataway, NJ) were incubated at 4°C overnight with the following antibodies: ERα (MC-20; Santa Cruz, 1:500), ERβ (Zymed; 1:100), and GAPDH (Chemicon; 1:2,000) in PBS-T (PBS, 0.02% Tween). The membranes were blocked in 5% milk in PBST (0.02% Tween-20) and incubated overnight at 4°C with the primary antibodies. Following incubation with the appropriate peroxidase-conjugated secondary antibody (Vector, 1:2,000), the immunoblots were developed using the enhanced chemiluminescence (ECI) plus Western blotting detection system (Amersham). Bands were scanned and analyzed densitometrically using National Institutes of Health (NIH) Image program tools. As a negative control, we used the Western blot using protein from the Chinese hamster ovary (CHO) cell line that lacks estrogen receptors.

Semiquantitative RT-PCR Analysis

For RT-PCR analysis, total RNA was isolated with the use of TRI Reagent (Sigma), with DNA removed by DNase 1 (Roche Diagnostics GmbH). Next, RNA was reverse-transcribed with Expand Reverse Transcriptase (Roche) in the presence of oligo-dT. cDNA was then amplified by polymerase chain reaction (PCR) with sets of nucleotides designed to recognize different isoforms of ERβ: ERβ1 and ERβ2: 5′ERβ: 5′ GAGCTCAGCCTGTTGGACCAA 3′; 3′ERβ: 5′GGCCTTCACACAGAGATACTCC 3′ (Maruyama et al., 1998); 5′ERβDBD: 5′ TCACTAGAGCACACCTTACC 3′; 3′ERβLBD: 5′ GCTGAGCAGATGTTCCATGC 3′, and ERα: 5′ GCTCCTAACTTGCTCTTGG 3′,5′ GCTGAAGTGGAGCTGGTGG 3′, as well as GAPDH: 5′ TGAAGGTCGGTGTCAACGGATTTGGC 3′; 5′CATGTAGGCCATGAGGTCCACCAC3′. Linear relationships between input template and the amount of amplification products were obtained.

RNase Protection Assay

The rat antisense ERβ probes recognizing isoforms differing in ligand-binding domain (LBD) were generated with the fol-

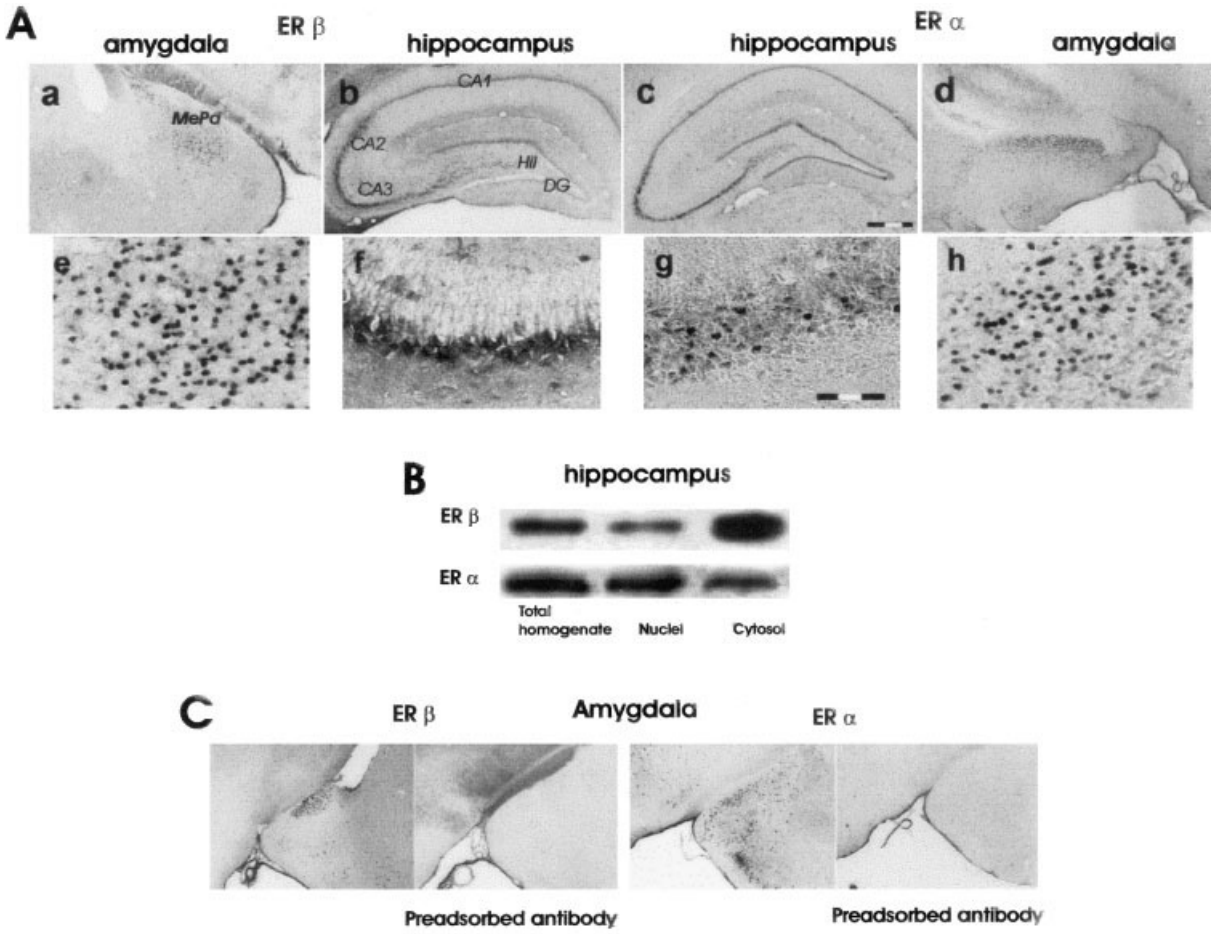

FIGURE 1. A: Expression of estrogen receptor β and α (ERβ, ERα) proteins in the adult rat brain. Coronal brain sections were stained immunocytochemically with either ERβ or ERα antibodies: ERβ (b) and ERα (c) immunoreactivity (ir) in the hippocampus; ERβ-ir (a) and ERα-ir (d) in the amygdala. High-magnification image of ERβ-ir (f) and ERα-ir (g) in the CA3 subfield. e,h: High-magnification ir image in medial amygdala. Hippocampal and amygdala pictures have been taken from the same brain section. Cytoarchitectural boundaries defined according to the rat brain atlas of Paxinos and Watson (1998). MePD, posterodorsal medial amygdala; Ca, cornu amonis; DG: dentate gyrus; Hil, hilus of dentate gyrus. B: Western blot analysis of expression of ERα and ERβ in subcellular fractions from hippocampal cells. ERα and ERβ presence in the total homogenate from the hippocampal cells vs. comparative expression of these receptor proteins in the nuclei and the cytosol. C: Immunohistochemistry with the ERβ or ERα antibodies and a corresponding staining with preadsorbed antibodies. Scale bars = 500 μm in Aa–d; 50 μm in Ae–h.

lowing vectors: pBluescript-ERβ2 (517–1030, Accession No. AF042059)-RPA LBD; pBluescript-ERβ2 (853–1306; Accession No. AF042059)-RPA 3; pBluescript-ERβ2 (989–1307; Accession No. AF042059) RNase protection assay (RPA)-RPA 4 (RPA 3; RPA 4); (Petersen et al., 1998). The RPA LBD produced a specific protected fragment of 509 bp and 434 bp; RPA 3: 454 bp and 296 bp; RPA 4: 315 bp and 269 bp. Total RNA was extracted with TRI Reagent (Sigma). Antisense riboprobes were generated from linearized templates using the Maxiscrip kit (Promega, Madison, WI), the appropriate RNA polymerase (T3 or T7), in the presence of ^{32}P-[CTP]. The radioactive probes were purified by agarose gel electrophoresis. Total RNA (20 μg for prostate and 100 μg for hippocampus) was hybridized with 5×10^5 cpm probe overnight in 50°C (Vladusic et al., 1998). Purified cRNA product was loaded on a 6% polyacrylamide gel in the presence of urea. Gels were exposed to autoradiographic film for 14 days.

RESULTS

Distribution of ERβ and ERα in the Hippocampus vs. Amygdala

To study the distribution of ERβ protein within the brain, we have followed an immunochemical approach. In a single male rat brain section, containing both amygdala and hippocampus, and using the same antibody, we have observed the diverse distribution of ERβ. A high level of nuclear ERβ immunoreactivity was detected in the medial amygdala (Fig. 1Aa,e), a brain structure previously suggested to be relatively abundant in this protein (Li et al., 1997; Greco et al., 2001; Mitra et al., 2003; Blurton-Jones and Tuszynski, 2002; Zhang et al., 2002). In addition, a nuclear localization of ERβ was observed in the sensory, perihinal, and enterohinal cortex and in the paraventricular nuclei. However, in the

hippocampal pyramidal cell layer of CA1–CA3 and in the hilar neurons of the dentate gyrus (DG), positive ERβ immunostaining was seen mainly in the cytoplasm of cell bodies, and not in the cell nuclei (Fig. 1Ab,f).

Using a similar immunochemical approach, with an antibody specific for ERα, we noted nuclear as well an extranuclear localization of this hormone receptor in the hippocampal formation. As in the case of ERβ, the same brain sections exhibited a different subcellular localization of ERα, depending on the brain region. What appeared as predominant nuclear localization of ERα was observed in the preoptic area, as well as in the medial and lateral amygdala (Fig. 1Ad,h). Within the hippocampal formation, ERα was present in the nuclei of cells in dendritic layers, along the border between the stratum radiatum and stratum lacunosum-moleculare, in the subiculum, and in the subgranular layer of the DG, with more cells stained in the suprapyramidal, rather than in the infrapyramidal blade (Fig. 1Ac). In the CA2–CA3 pyramidal cell layer, the ERα signal also localized to the perinuclear regions. (Fig. 1Ag).

The same approach was followed in the brains of intact female rats of the same age. The results, with regard to the intracellular appearance of ERα and ERβ in the hippocampus and the amygdala, were similar to those for the male rats.

No evident nuclear or cellular specific labeling was found in the control experiments, in which either the primary or secondary antibody was omitted from the immunolabeling protocol. In addition, we have carried out preadsorbtion of the primary antibodies for ERα and ERβ with their blocking peptides. No labeling was visible on the sections for either protein when antibodies have been preadsorbed, attesting to the specificity of the selected antibodies (Fig. 1C).

Subcellular Localization of ERα and ERβ Proteins

To analyze further the subcellular localization of ERα and ERβ proteins in the hippocampus, we have carried out a series of cell fractionations, resulting in separation of the nuclei from the cytosol. The presence of ERβ was abundant in the cytosol, a fraction devoid of most organelles, such as the nuclei, mitochondria, vesicles, and membranes (Fig. 1B). In contrast, ERα was found to be much more abundant in the nuclei and much less abundant in the cytosol (Fig. 1B).

ERβ Protein Expression in Hippocampus and Medial Amygdala

The level of ERβ in total protein extracts from the hippocampus and the medial amygdala was analyzed by Western blot assay. The results showed no difference in the level of ERβ protein expression between the two structures analyzed (Fig. 2A), as compared with the housekeeping control protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Furthermore, the ERβ proteins identified in the amygdala and hippocampus displayed identical gel mobility. Specificity of the antibody employed was confirmed by demonstrating its interaction with a protein extracted from yeast bearing rat ERβ cDNA, encoding a slightly truncated protein (Fig. 2A), in addition to incubating blotted proteins with a preadsorbed antibody (data not shown).

Expression of ERβ mRNA Isoforms in the Hippocampus and Medial Amygdala

The fact that the intracellular distribution of ERβ protein is different in the hippocampus and medial amygdala raises a question as to whether these marked differences in protein localization correlate with a different profile of mRNA expression in the particular brain structures. To test this possibility, we have developed an RPA to define the ERβ mRNA isoform profile in the hippocampus. To compare this result with a well-established source of ERβ mRNA, we used prostate tissue in parallel (Fig. 2B, lane 1). We have found that the ERβ1 and ERβ2 comprise the major ERβ mRNAs expressed in the rat hippocampus (Fig. 2B, lanes 2). Following this information, we designed appropriate (PCR) primers to analyze these two isoforms, employing a lower amount of mRNA than was available for individual animals, but still sufficient for reverse transcription (RT)-PCR.

To establish optimal PCR assay conditions for the cDNA, linearity of the ERβ amplification protocol was assessed by running 20, 25, 30, 35, 40, and 45 cycles, using hippocampal material. A strong linearity for ERβ1 and ERβ2 mRNAs was demonstrated within a range of 25–35 cycles; 35 cycles were applied for all further semiquantitative RT-PCR measurements. The amount of PCR product was quantified and compared with the level of GAPDH product amplified from the same cDNA sample (Fig. 2C). The results of the RT-PCR experiment demonstrate no significant difference in the expression of major ERβ1 and ERβ2 isoforms in the hippocampus versus the medial amygdala (Fig. 2C).

Colocalization of ERβ With Neuronal Markers in the Hippocampus

Double-label immunohistochemistry for estrogen receptors and neuron- or glia-specific marker proteins, NeuN and GFAP, respectively, has revealed the dominant presence of ERβ in the neurons of the rat hippocampus, rather than in glial cells (Fig. 3a and b, respectively). In an attempt to identify the nature of the ERβ-carrying neurons in the hippocampus, double-label immunohistochemistry was carried out for the ERβ and an interneuron-specific parvalbumin protein or a γ-aminobutyric acid (GABA) ergic inhibitory neuronal marker (GAD65/67), or a marker of excitatory neurons: α subunit of type II Ca^{2+}/calmodulin-dependent protein kinase (αCamKII). Very few cells in the hippocampus were positive for ERβ and PV, and most of the parvalbumin-positive inhibitory interneurons did not express that estrogen receptor (Fig. 3c). We did not observe colocalization of ERβ with GAD65/67, the GABAergic inhibitory neuronal enzyme (Fig. 3d). However, we noted a clear colocalization of the ERβ with the α subunit of αCamKII (Fig. 3e,f).

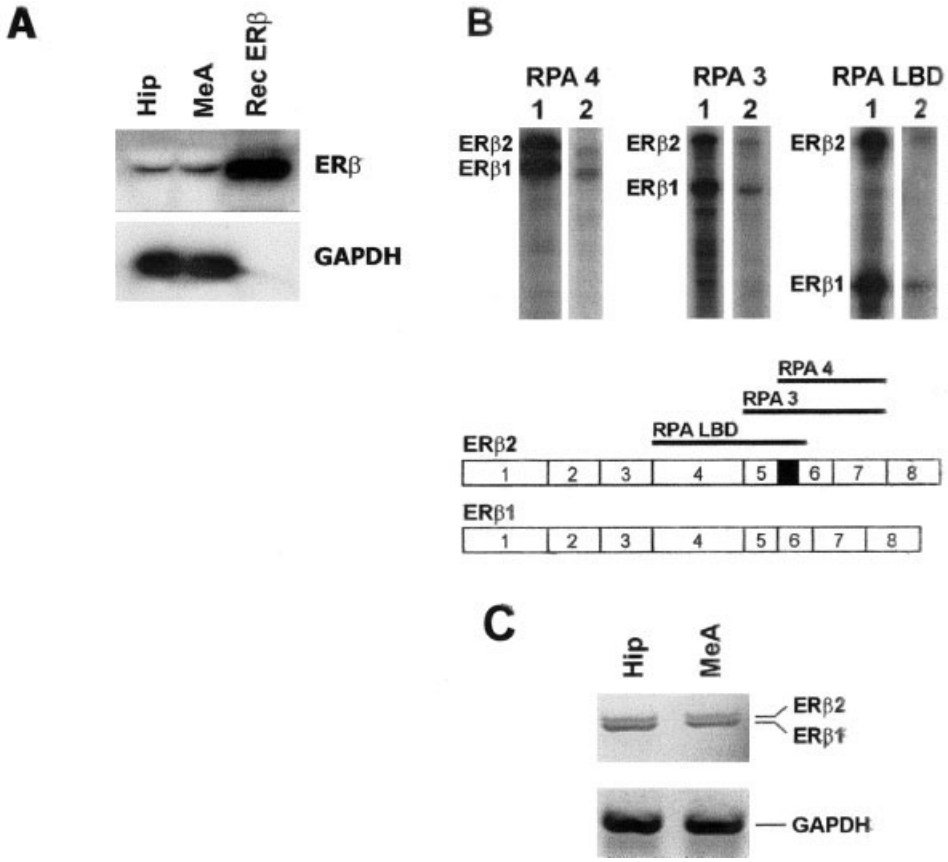

FIGURE 2. A: ERβ protein expression in the rat hippocampus (Hip) and medial amygdala (MeA) as detected by immunoblotting. Protein lysates were prepared from tissue of male rats and analyzed with the ERβ antibody. In both cases, a single band of molecular weight ~60 kDa was observed that was similar to the mobility of the recombinant, truncated ERβ rat protein expressed in yeast (recERβ). There is no difference in the level of ERβ protein expression between Hip and MeA. The samples contained equal amounts of total protein as indicated by the levels of GAPDH. B: Analysis of alternatively spliced transcripts of ERβ. Analysis of ERβ transcripts in the adult hippocampus, and prostate (see Results) by RNase protection assay (RPA). Radioactive cRNA probes covering different parts of ERβ transcripts, i.e., RPA ligand-binding domain (LBD), RPA 3, and RPA 4, were used to differentiate among the mRNA species. Amount of total RNA loaded in lane 1 (prostate), 20 μg; lane 2 (hippocampus), 100 μg. The samples were exposed to X-ray film for 14 days. C: RT-PCR analysis of expression of ERβ1 and ERβ2 mRNA isoforms in the rat hippocampus (Hip) and medial amygdala (MeA). PCR primers were designed to recognize the two transcripts in one PCR reaction. The estimated products length is 1103 bp for ERβ1 and 1157 bp for ERβ2. cDNA from male hippocampus and medial amygdala was used as a template. GAPDH mRNA analysis was performed to verify equal reaction and loading.

DISCUSSION

The expression of ERβ in the hippocampus of hormonally intact male and female rats is demonstrated and data are presented to show that this receptor differs from ERα, with respect to both its level of expression and distribution within the hippocampus, as well as its subcellular localization. Our major findings may be summarized as follows: (1) ERβ displays nuclear localization in the amygdala and extranuclear localization in the hippocampus; and (2) the extranuclear ERβ localizes to the cell cytosol and resides in excitatory neurons, particularly in the CA3 subfield of the hippocampus.

The original reports of in vivo estrogen binding in the hippocampus (Pfaff and Keiner 1973; Stumpf and Sar, 1975) were performed with [^{3}H]estradiol. The sensitivity of this method is low and produced only sparse binding in the hippocampus. Estrogen radiolabeled with ^{125}I-estradiol (Shughrue and Merchenthaler, 2000) revealed many more estrogen binding sites in rat hippocampus (CA1–CA3 and dentate gyrus). Corresponding to the high levels of estrogen binding, and in support of our findings, several other investigators have also demonstrated a large number of ERα- and ERβ-labeled hippocampal neurons. Li et al. (1997) detected high levels of ERβ immunoreactivity in the CA1, CA2, and dentate gyrus of the hippocampal formation, as well as in the medial amygdala, noting non-nuclear immunoreactivity in the hippocampal pyramidal cells. Immunohistochemistry staining by Hart et al (2001) revealed many more ERα-labeled cells in the hippocampus than were observed in previous studies. In addition, Kritzer (2002) found immunoreactivity for the ERβ to be sensitive to the conditions of fixation, where it is markedly attenuated in fixatives containing 4% paraformaldehyde (with and without acrolein) and considerably stronger in more dilute aldehyde fixatives (1–2%). This has also been our observation.

Somewhat surprisingly, in the hippocampus, we have observed similar levels of the ERβ mRNA and protein as in the amygdala,

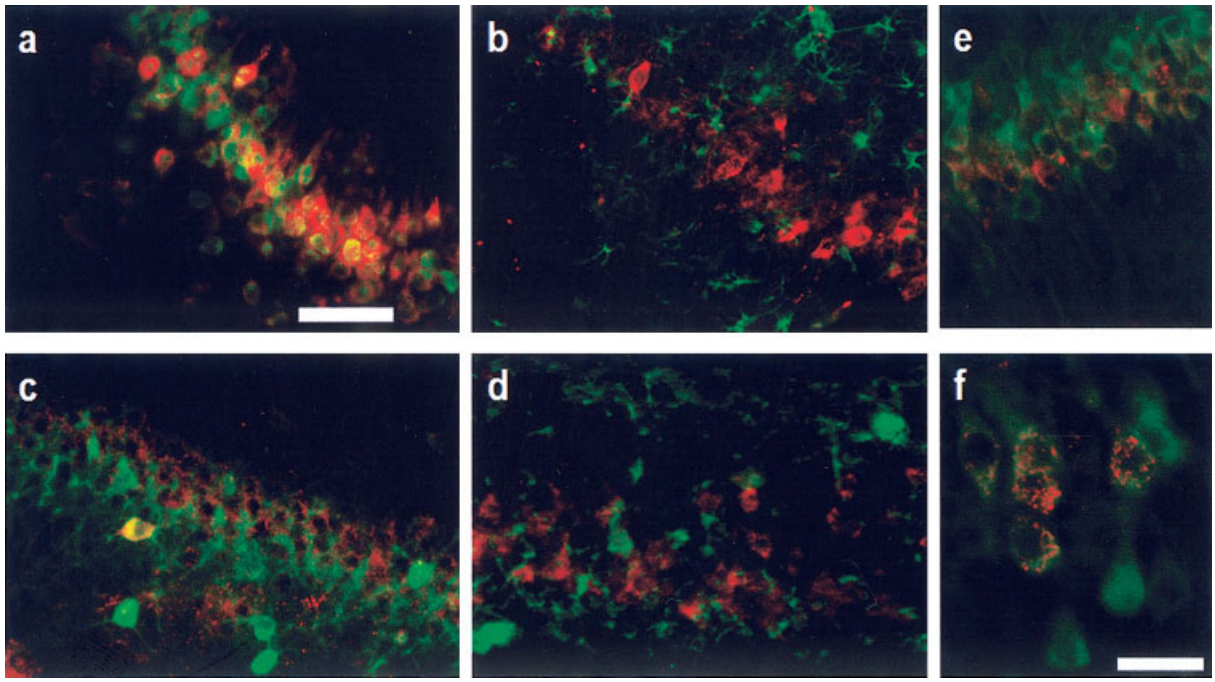

FIGURE 3. Double-label immunohistochemistry for ERβ (red) and either neuronal or glial markers (green) in the CA3 pyramidal cell layer of hippocampus. a: ERβ and NeuN. b: ERβ and GFAP protein. c: ERβ and parvalbumin. d: ERβ and GAD65/67-expressing inhibitory neurons. e,f: Double staining for ERβ and αCamK II. Scale bars = 50 μm in a–e; 20 μm = f.

where the protein was highly abundant in the nuclei. To ensure the specificity of the anti-ERβ antibody, we have analyzed the molecular size of the immunodetected protein band along with a recombinant protein produced in yeast, in addition to carrying out immunohistochemistry reactions with antibodies to ERβ and ERα that were preadsorbed with the immunization peptide. Thus, we believe that we have veridically detected the ERβ mRNAs and protein in the rat hippocampus, despite an unusual, extranuclear localization of this transcription factor protein.

Previous studies on the distribution and abundance of expression of ERα and ERβ have indicated that aside from some overlap, there are many differences in the expression of these two receptors (Shughrue et al., 1997). In our work, we have further explored these differences between ERα and ERβ in an effort to find novel data regarding their subcellular distribution. We note that ERβ localizes predominantly to the cytosol portion of hippocampal cells, whereas ERα can be found primarily in the cell nuclei. Extranuclear location of estrogen receptors has been reported by Monje and Boland (2001), who, in estrogen-responsive tissue, have demonstrated the presence of ERα and ERβ as well as the binding of radiolabeled estradiol in the mitochondrial, cytosolic, microsomal, and membrane fractions, in addition to the nucleus. Biochemically functional estrogen receptors present in the cell cytosol were initially reported by Jakesz et al. (1983), who analyzed the estrogen-binding properties of cytosol and nuclear populations of the estrogen receptor. The presence and activity of estrogen receptors have also been demonstrated at the plasma membrane using (BSA)-conjugated estradiol (Ramirez et al., 1996; Kuroki et al., 2000), while conventional, confocal (Clarke et al., 2000), and immunoelectron microscopy (Milner et al., 2001; Adams et al., 2002) were used to localize the ERα to non-nuclear sites.

Subcellular analysis of ERβ on the ultrastructural level has apparently not yet been carried out; however, Mitra et al. (2003) have localized extranuclear ERβ in the CA subfields of mouse hippocampus. In recent work, Yang et al. (2004) demonstrated specifically that ERβ localizes to the mitochondria in cardiomyocytes as well as cultured hippocampal neurons. The investigators have supported their immunocytochemistry results with mass spectrometry, in which they have positively identified the ERβ among mitochondrial proteins. The subcellular localization of ERβ in mouse hippocampus was investigated by Nishio et al. (2004), who observed this steroid receptor predominantly in nuclear, synaptosomal, and synaptic membrane fractions. A change in the compartmentalization of the ERβ (i.e., a shift from the nucleus to the synaptic membrane) was reported by Lu et al. (2004), who suggested that it could play a role in the pathogenesis of Alzheimer's disease and that it may represent an important regulator of intracellular signal transduction from membrane to cytosol in hippocampal neurons. In another study, ERβ was found in the aged human hippocampus and immunolocalized within the perinuclear cytoplasm of the DG and the pyramidal layer of CA1–CA3 (Savaskan et al., 2001). This is comparable to our results with ERβ staining in the rat, where we note its cytoplasmic distribution within the pyramidal cells of CA1–CA3 and the hilus of the dentate gyrus. This finding was confirmed by results obtained from differential centrifugation, where we repeatedly find the greatest concentration of ERβ in the cytosol-containing fraction. By comparison, ERα was poorly expressed in that fraction. Such subcel-

lular localization differences combined with various molecular studies (Ogawa et al., 1998; Hall et al., 2002) suggest that ERα and ERβ can play different roles in the regulation of neuronal physiology, particularly with respect to participation in nongenomic mechanisms.

Recent studies have reported actions of estrogens that appear to be too rapid to be engaging transcription regulation, and that involve the stimulation of various second messenger systems in the cytoplasm (Kelly and Wagner, 1999; Singh et al., 2000). The rapid effects of estrogens are most probably mediated by non-nuclear ERs or other membrane receptors. It has been demonstrated that expression of cDNAs for ERα and ERβ in CHO cells (which normally do not produce ER) results in the presence of both membrane and nuclear pools of these receptors (Razandi et al., 1999). Thus, it is probable that the non-nuclear receptor may be the same protein as the nuclear one, but translocated to another cell compartment (e.g., due to various posttranslational modifications such as, palmitylation or myristylation) and capable of expressing nongenomic abilities.

Contrary to a report by Garcia-Ovejero et al. (2002), in which both forms of estrogen receptor were found mostly in glial cells, particularly after hippocampal lesions, our work has found the ERβ as well as ERα to be expressed in the hippocampal neurons of a healthy rat. This expression is evident primarily in the CA subfields. We also show that neurons expressing ERβ in the CA subfields and the dentate gyrus do not coexpress glutamic acid decarboxylase (GAD) or parvalbumin, a calcium-binding protein, both of which indicate GABAergic interneurons. Such a co-localization of ERβ and inhibitory interneuronal markers in the pyramidal layer of the subiculum was found by Blurton-Jones and Tuszynski (2002). In contrast, we find ERβ to be colocalized with αCaKII in the CA3 subfield, indicating its presence in the excitatory pyramidal neurons. This finding raises the possibility that estrogen should be able to act directly through its receptors present in the excitatory pyramidal cells.

Studies of brain estrogen receptors have concentrated primarily on the female of the species. Yet both sexes possess estrogen receptors, the function of which, particularly in the hippocampus, is most likely unrelated to reproduction. To assess whether our observations regarding the intracellular localization of the ERα and ERβ in the hippocampus are comparable between males and females, we have also carried out immunostaining on brain sections from intact female rats. We found no significant differences in the expression of both receptors between the sexes. Studies comparable to ours, which have included either males only or both sexes, (Weiland et al., 1997; Garcia-Ovejero et al., 2002; Zhang et al., 2002), report localization of ERα and ERβ in the rat brain to be similar to the results discussed in the present report. Although there are some differences between males and females as far as the expression of estrogen receptors in the rat brain (Yokosuka et al., 1997; Zhang et al., 2002; Perez et al., 2003), these differences are mostly quantitative. The exceptions are the sexually dimorphic areas, which in the case of ERβ, include the medial mammillary nucleus, the caudal part of the pontine resticular nucleus, subceruleus (only in males), and the superior vestibular nucleus (only in females).

In our work, we have looked only at the dorsal portion of the rostral hippocampal formation starting at bregma: −2.3 to −4.5 and the corresponding amygdala region, however, rostrocaudal observations have been made by other investigators. According to Shughrue et al. (1997) ERβ mRNA is expressed throughout rostrocaudal extent of the hippocampus. Hart et al. (2001) show the ERα protein to be expressed throughout rostrocaudal hippocampus. Mitra et al (2003) report darker and more nuclear staining for ERβ in the caudal hippocampus, while observing stronger ERα labeling in the ventral portion of hippocampus than in the dorsal. Both receptors colocalize in the medial amygdala (Shughrue et al., 1997) but, while the ERα was found to be abundant throughout the extent of that structure, the ERβ-ir cells were most heavily concentrated in the caudal portion of medial amygdala (Mitra et al., 2003; Greco et al., 2001).

In conclusion, it may be stated that the two estrogen receptor subtypes differ with respect to their subcellular distribution in the rat hippocampal formation. That observation, together with their differential distribution and level of expression throughout the brain, suggests that ERα and ERβ participate in different intracellular mechanisms and support different brain functions. In the aggregate, our results and the aforementioned considerations raise the possibility that cytoplasmic estrogen receptors may play an important role in hippocampal physiology.

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