0013-7227/03/$15.00/0
Printed in U.S.A.

Endocrinology 144(5):2055–2067

doi: 10.1210/en.2002-221069

Immunolocalization of Estrogen Receptor β in the Mouse Brain: Comparison with Estrogen Receptor α

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Estrogen receptor α (ERα) and ERβ are members of the steroid nuclear receptor family that modulate gene transcription in an estrogen-dependent manner. ER mRNA and protein have been detected both peripherally and in the central nervous system, with most data having come from the rat. Here we report the development of an ERβ-selective antibody that cross-reacts with mouse, rat, and human ERβ protein and its use to determine the distribution of ERβ in the murine brain. Further, a previously characterized polyclonal antibody to ERα was used to compare the distribution of the two receptors in the first comprehensive description of ER distribution specifically in the mouse brain. ERβ immunoreactivity (ir) was primarily localized to cell nuclei within select regions of the brain, including the olfactory bulb, cerebral cortex, septum, preoptic area, bed nucleus of the stria terminalis, amygdala, paraventricular hypothalamic nucleus, thalamus, ventral tegmental area, substantia nigra, dorsal raphe, locus coeruleus, and cerebellum. Extranuclear-ir was detected in several areas, including fibers of the olfactory bulb, CA3 stratum lucidum, and CA1 stratum radiatum of the hippocampus and cerebellum. Although both receptors were generally expressed in a similar distribution through the brain, nuclear ERα-ir was the predominant subtype in the hippocampus, preoptic area, and most of the hypothalamus, whereas it was sparse or absent from the cerebral cortex and cerebellum. Collectively, these findings demonstrate the region-selective expression of ERβ and ERα in the adult ovariectomized mouse brain. These data provide an anatomical framework for understanding the mechanisms by which estrogen regulates specific neural systems in the mouse. (*Endocrinology* 144: 2055–2067, 2003)

ESTROGEN plays a critical role in the development, maintenance, and physiology of male and female reproductive tissues as well as nonreproductive systems, including the cardiovascular, skeletal, and central nervous systems (CNS) (1). Estrogen is believed to mediate its effects by binding to the products of two related genes, estrogen receptor α (ERα) and ERβ, members of the steroid receptor branch of the nuclear receptor superfamily. ERs, when bound to agonists such as 17β-estradiol, dimerize and interact with regulatory DNA sequences to modulate gene transcription by interacting with coactivators and the basal transcriptional machinery (2, 3). Further, increasing evidence indicates rapid, nongenomic actions of estrogen, and some of these effects may be mediated through extranuclear ERα and/or ERβ (4–8).

The CNS is a major target of estrogen action (9, 10), yet the mechanisms by which estrogen mediates these effects are not clear. Knowledge of the distribution of ERs across specific brain regions provides insight into estrogen target sites and the complex regulatory actions this class of steroid hormones has on brain function. To date, the most extensive information regarding ER expression patterns in the CNS has come specifically from the rat (11–18). A comprehensive study comparing ERα and ERβ mRNA expression patterns in rat CNS has indicated some overlap, but also some striking differences in the distribution and abundance of expression of these two receptors (14). Molecular studies suggest that the two ER isoforms can play different roles in estrogen regulation of gene expression and neuronal physiology (2, 19–22). Further, a comparison of ER mRNA expression patterns in rat (11, 14, 23), mouse (24, 25), monkey (26), and human (27, 28) indicates that data cannot be extrapolated from one species to another.

The mouse has become an increasingly valuable species to identify specific molecular mediators of steroid hormone actions, largely due to the availability of transgenic/knockout models (29, 30). Shughrue and colleagues (24, 31) were the first to report ERβ mRNA and [^{125}I]estradiol binding specifically in the forebrain of the ERα knockout (αERKO) mouse as evidence for the expression of functional ERβ protein in select forebrain regions. However, this group has more recently identified low levels of estrogen binding and regulation of gene expression in the preoptic area of the double αβERKO mouse (32), activity attributed to an ERα splice variant specifically produced in the αERKO and previously identified in uterus (33). Collectively, these findings underscore the importance of using subtype-selective antibodies to confirm the distribution of ERα and ERβ protein in the mouse brain.

The ERα gene was cloned 10 yr before the identification of ERβ (34, 35); thus, many more antibodies to the ERα have been generated (36). In the present study we describe the

Abbreviations: aa, Amino acid; BNST, bed nucleus of the stria terminalis; CNS, central nervous system; ER, estrogen receptor; αERKO, ERα knockout; -ir, immunoreactivity; PB, sodium phosphate buffer; PVN, paraventricular nucleus; TSA, tyramide signal amplification.

characterization of a polyclonal peptide antibody (80424) raised against a conserved region of the human, mouse, and rat ERβ protein recently used to identify a longer isoform of human ERβ-548 (37). Using the 80424 antibody, we describe the distribution of ERβ immunoreactivity (-ir) in the mouse brain. Further, a previously characterized antibody to ERα (AS409; Refs. 38–41) was used to compare the distributions of the two receptor subtypes in the ovariectomized mouse brain.

Materials and Methods

Development of ERβ antibody 80424

Rabbits were immunized with the peptide (EARSLEHTLPVNRETLKRK)-8-MAP representing amino acid (aa) residues 64–82 of ERβ-485 (accession no. U57439; spanning exons 2 and 3), and serum was collected and titrated by Research Genetics, Inc. (Huntsville, AL). Antibodies were affinity-purified over Amino-Link columns (Pierce Chemical Co., Rockford, IL) conjugated with the immunogen and eluted with 0.1 M glycine, pH 3. Eluates were dialyzed against PBS and kept frozen in aliquots at –80 C.

Immunoblotting

Extracts of SF9 cells expressing rat ERα (595 aa), human ERα (595 aa), mouse ERβ (485 aa), rat ERβ (485 aa), human ERβ (485 aa), and ERβ (530 aa) were obtained from Stefan Nilsson (Karo Bio, Huddinge, Sweden). Human tissues were obtained from the National Disease Research Interchange (Philadelphia, PA). Extracts from tissues and cells were reduced and denatured in SDS/mercaptoethanol by heating to 75 C for 20 min, then subjected to SDS-PAGE using ready made gels [Bio-Rad Laboratories, Inc. (Hercules, CA) and Novex (San Diego, CA)]. Separated proteins were transferred to polyvinylidene difluoride membranes by electrophoresis and blocked with blocking buffer [10 mM phosphate (pH 7.5), 0.1 M NaCl, 10% glycerol, 10% nonfat milk, and 0.2% Tween 20]. Blots were incubated at room temperature with the antibody at a 1:2000 dilution for 16 h. Unbound antibody was removed by repeated washing with blocking buffer, and cells were treated with horseradish peroxidase-conjugated antirabbit antibody (Sigma-Aldrich, Milwaukee, WI) at a 1:2000 dilution for 1 h at room temperature. The blots were developed with Supersignal West Dura ECL substrates (Pierce, Rockford, IL) according to the manufacturer's instructions. Total extracts from mouse and rat brain and human ovary and testes were immunoblotted with a 1:200 dilution of antibody. Peptide competition was performed by incubating 200 ng peptide immunogen/μl antibody for 1 h at room temperature before use.

Immunofluorescence microscopy

COS-7 cells were transiently transfected with ERα and ERβ to initially determine the sensitivity and selectivity of 80424 in immunocytochemistry. Transfected COS-7 cells were fixed with 3% paraformaldehyde in PBS. Cells were permeabilized with 1% Triton X-100 in PBS, and nonspecific binding was blocked with normal goat serum. Cells were incubated in the anti-ERβ primary antibody, 80424, at a 1:1000 dilution. Fluorescein isothiocyanate-conjugated antirabbit antibody was used as the secondary antibody at a dilution of 1:2000. Slides were examined under a Nikon light microscope (Melville, NY), and images were captured with a Hamamatsu digital camera (Hamamatsu City, Japan) and Metamorph software (Universal Imaging Corp., Downingtown, PA).

Animal treatment and tissue preparation

Animal care, maintenance, and surgery were performed in accordance with regulations dictated by the Animal Welfare Act and the U.S. DHHS Guide for the Care and Use of Laboratory Animals. Young adult female mice derived from crosses of C57BL/6J and 129 ancestry were ovariectomized under Metofane anesthesia. Two weeks later, animals were deeply anesthetized with Metofane and were transcardially perfused with 100 ml 3.75% acrolein and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (PB), pH 7.4. Brains were immediately removed and postfixed in 2% paraformaldehyde in 0.1 M PB at 4 C overnight. Brains were transferred into 30% sucrose in 0.1 M PB, frozen on dry ice, and sectioned at 30 μm on a sliding microtome. Sections were stored in cryoprotectant (30% glycerol and 30% ethylene glycol in 0.1 M PB, pH 7.4) at –20 C until processing for immunocytochemistry.

Immunocytochemistry

Free-floating, 30-μm sections were washed in cold 0.1 M PB with saline (PBS), pH 7.4, to thoroughly remove cryoprotectant. To ensure consistent immunolabeling across animals, a screen-bottom, 24-well tissue holder and fitted solid Lucite tray containing buffer were used. Thus, all sections were coincubated in the same solutions/conditions during the entire experiment. To deter nonspecific staining in acrolein-fixed tissue (remove excess aldehyde groups), sections were washed in 1% sodium borohydride ($NaBH_4$) in PBS for 30 min and rinsed 8–10 times with PBS. Endogenous peroxidase activity was inhibited by washing sections in 0.3% hydrogen peroxide and 20% methanol in PBS for 15 min. Sections were washed several times in PBS and then blocked with 2% normal serum in PBS with 0.2% Triton X-100 for 1 h. Sections were incubated with the rabbit polyclonal ERβ antibody (80424; 1:14,000; stock, 4.8 mg/ml) over 3 nights in blocking buffer diluted to 1% normal serum and 0.1% Triton X-100 at 4 C. Several controls were included to ensure the specificity of the immunocytochemical labeling with the 80424 antibody. A subset of sections was incubated 1) in primary antisera that had been preabsorbed with the antigenic synthesizing peptide (1:1, wt/vol); 2) in antibody preabsorbed with a nonantigenic peptide sequence of the ERβ (1:1, wt/vol), or 3) without primary antibodies. Adjacent sections were incubated with an extensively characterized rabbit polyclonal antibody to ERα (AS409; 1:20,000) (38–41) over 5 nights to compare the distribution of ERα. We recently demonstrated the specificity of this antibody for the ERα in mouse brain; no immunolabel was observed in the αERKO dorsal raphe (40), a region with abundant nuclear ERβ. Sections were washed in PBS and exposed to a biotinylated secondary goat antirabbit antibody (1:600; Vector Laboratories, Inc., Burlingame, CA) in PBS with 0.2% Triton X-100 and 1% normal serum for 1 h. After several PBS washes, tissue was exposed to the avidin-biotin complex (ABC Elite kit, Vector Laboratories, Inc.) in PBS for 30 min. Sections were rinsed in PBS and incubated in tyramide signal amplification (TSA) solution (1:50; NEN Life Science Products, Boston, MA) for 10 min, washed in PBS, and then incubated with streptavidin-horse radish peroxidase (1:200) for 30 min. A subset of tissue was not incubated with the TSA system for comparison. Sections were washed in PBS, followed by 0.175 M sodium acetate buffer (pH 7.0; Sigma-Aldrich, St. Louis, MO), and then exposed to the substrate 3,3′-diaminobenzidine tetrachloride containing nickel sulfate and hydrogen peroxide in 0.175 M sodium acetate buffer for 3 min. The reaction product appears as a dark blue-black punctate stain, primarily in cell nuclei. After one sodium acetate buffer rinse and several PBS washes, sections were mounted onto gelatin-coated slides in 0.05 M PB, air-dried overnight, dehydrated in ascending ethanol concentrations, cleared in xylene, and coverslipped with DPX mounting medium. Photographs were taken under a Nikon light microscope and 35-mm camera (Nikon, Tokyo, Japan) using Kodak Ektachrome slide film (Eastman Kodak Co., Rochester, NY). Slides were scanned and saved to a Macintosh computer (Cupertino, CA), imported into Adobe Photoshop (Adobe Systems, Mountain View, CA) for labeling.

Tissue analysis

Sequential 30-μm sections, approximately 180 μm apart, from the olfactory bulbs through the hindbrain were immunostained as described above. Each section was anatomically categorized according to distance from bregma (B) using *The Mouse Brain in Stereotaxic Coordinates* (42) as a guide. The distribution and relative density of cells immunoreactive to the 80424 antibody (ERβ) or AS409 (ERα) were documented at magnifications of ×100 and ×200 using a Nikon light microscope. Data were expressed in tabular form: −, no ER-ir cells; −/+, few ER-ir cells; +, ER-ir cells present; 2+, abundant ER-ir; and 3+, very abundant ER-ir. Maps were generated to depict the distribution of nuclear 80424 (ERβ)-ir, as ERβ localization with this affinity-purified antibody was the primary focus of this study.

Results

Antibody specificity

To generate an ERβ-selective antibody, we examined an alignment of deduced aa sequences of the mouse, human, and rat ERβ proteins that revealed a conserved region located at aa 64–82 of rat ERβ (485 aa) (Fig. 1A). This sequence is located within the A/B domain of ERβ (exons 2–3) and is not conserved in ERα. Accordingly, this peptide was selected to generate rabbit polyclonal antibodies that would be subtype selective, but not species specific. The serum from one rabbit (designated 80424) was peptide affinity-purified and used for the analyses described below.

Immunoblotting total extracts of SF9 cells transfected with cloned rat and mouse (Fig. 1B, lanes 1 and 2) and human ERβ (Fig. 1C, lanes 1 and 2) identified a band migrating at approximately 60 kDa, whereas no staining was detectable in extracts of cells transfected with either rat or human ERα (Fig. 1B, lanes 3 and 4). A band migrating at approximately 55 kDa was detected in extracts of human ovary and human testes (Fig. 1C, lanes 3 and 4). The latter showed an additional band migrating with a mobility of approximately 70 kDa. Upon immunoblotting whole tissue extracts from the rat and mouse brain, a single major band migrating with the mobility of about 70 kDa was detected (Fig. 1C, lanes 5 and 6). No bands were detected in tissue extracts or in extracts of SF9 cells expressing cloned mouse, rat, or human ERβ when 80424 was preincubated with the immunogenic peptide (Fig. 1B, lanes 5 and 6, and Fig. 1C, lanes 7–12). Therefore, 80424 selectively recognizes this peptide sequence in mouse, rat, and human ERβ and proteins of similar size in whole tissue extracts.

A population of the COS-7 cells transiently transfected with human ERβ showed strong nuclear staining by immunofluorescence with the 80424 antibody, and this labeling could be eliminated by prior incubation with the immunogenic peptide (Fig. 2). No staining was detectable in ERα-transfected cells or in cells transfected with an empty vector (data not shown). Therefore, 80424 was determined to be suitable for immunocytochemical studies.

ERβ (80424-ir) localization in mouse brain

The specificity of the 80424 antibody in mouse brain tissue is demonstrated in Fig. 3. The majority of immunostaining was detected specifically within cell nuclei, as demonstrated in the locus coeruleus (Fig. 3, A and C); however, cells within select regions consistently exhibited extranuclear (somal and/or fiber) labeling (Fig. 3, D and F). Importantly, no staining was present when tissue was incubated with antibody that had been preadsorbed with the antigenic peptide (Fig. 3, B and E). When the 80424 antibody was preincubated

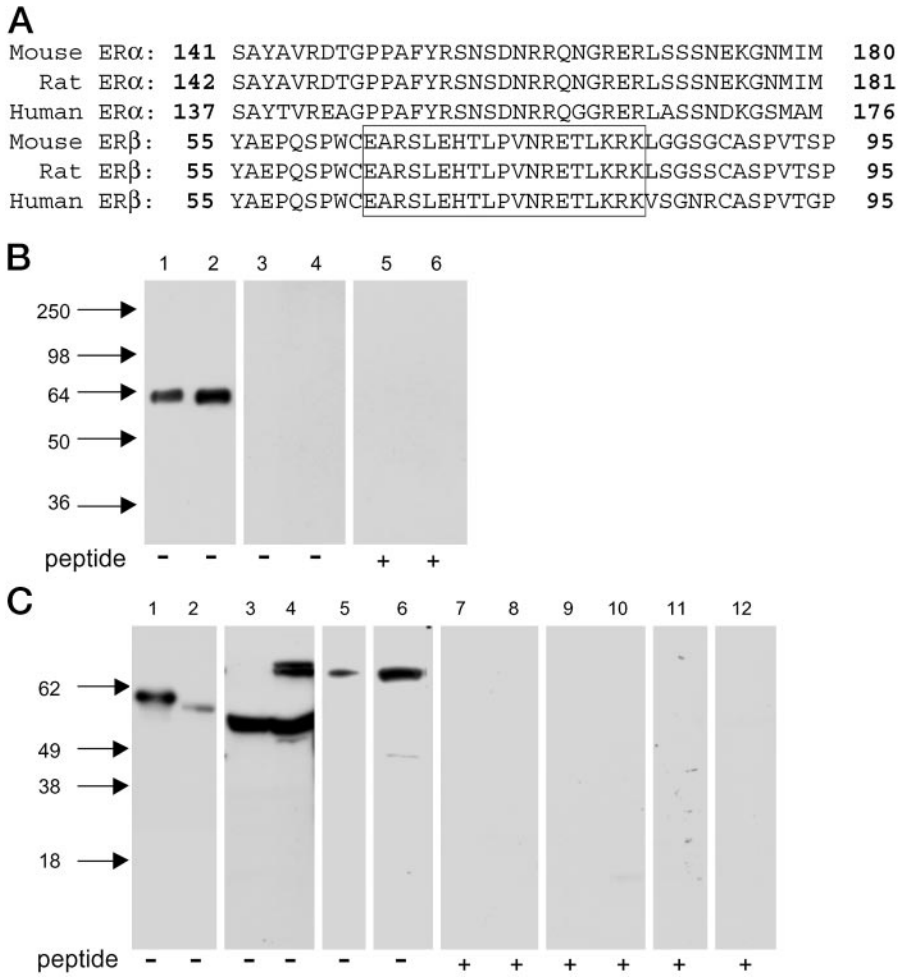

FIG. 1. Development and characterization of the ERβ selective antibody, 80424. A, Alignment of conserved aa sequences at the 5′ end of mouse, rat, and human ERβ and comparison of aa sequences in the corresponding region of mouse, rat, and human ERα. B, Determination of selectivity of 80424 by immunoblot analysis. Extracts of SF9 cells overexpressing ERβ from mouse (lanes 1 and 5) and rat (lanes 2 and 6) and ERα from rat (lane 3) and human (lane 4) were separated by SDS-PAGE and immunoblotted with either 80424 or 80424 preincubated with immunogenic peptide (lanes 5 and 6). C, Demonstration of cross-species reactivity with 80424. Extracts of SF9 cells overexpressing human ERβ-530 (lanes 1 and 7), human ERβ-485 (lanes 2 and 8), human ovary (lanes 3 and 9), human testes (lanes 4 and 10), mouse brain (lane 5 and 11), and rat brain (lane 6 and 12) were separated by SDS-PAGE and immunoblotted with 80424 (1–6) or with 80424 that had been preincubated with the immunogenic peptide (lanes 7–12).

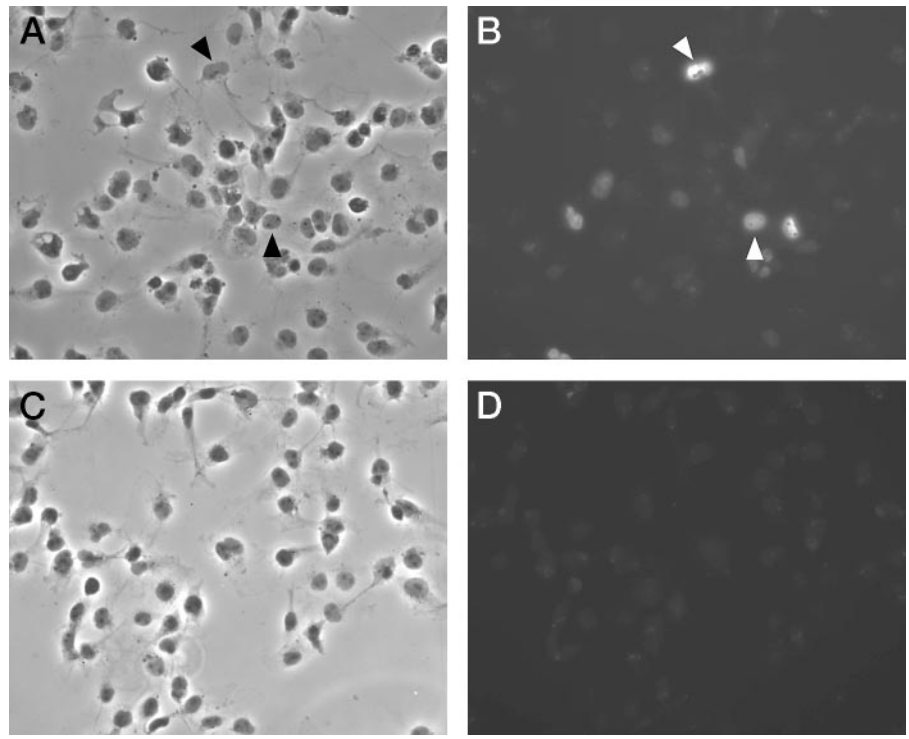

FIG. 2. Immunofluorescent staining of COS-7 cells overexpressing ERβ by 80424. COS-7 cells transiently transfected with human ERβ were fixed and incubated with either 80424 (A and B) or 80424 preincubated with immunogenic peptide followed by fluorescein isothiocyanate-labeled goat antirabbit antibody (C and D). A and C, Corresponding brightfield images.

with a peptide from an alternate region of ERβ, immunolabeling was indistinguishable from that seen in tissue incubated with antibody alone (Fig. 3C). The TSA system enhanced signal detection.

Immunostaining with the 80424 antibody demonstrated a region-specific distribution of ERβ protein through the rostral-caudal extent of the ovariectomized adult mouse brain. Brain maps depicting the distribution and relative expression level of nuclear ERβ-ir are shown in Fig. 4. Table 1 is a comprehensive list of the specific brain regions observed to exhibit ERβ-ir and/or ERα-ir and the subcellular distribution of immunolabel.

Forebrain. Forebrain regions found to exhibit the greatest concentration of intense ERβ-ir included the vertical and horizontal limbs of the diagonal band, bed nucleus of the stria terminalis (BNST), medial amygdala (particularly the caudal extent), magnocellular preoptic nucleus, and dorsomedial, medial tuberal, and supramammillary nuclei of the hypothalamus. Strong to moderate nuclear ERβ-ir was observed in cells of the olfactory bulb, claustrum, medial septum, globus pallidus, preoptic area, select regions of the cerebral cortex (particularly along the external capsule, piriform, agranular insular, and entorhinal cortexes), basal nuclei of the amygdala, paraventricular (PVN) nucleus of the hypothalamus (magno- and parvicellular divisions), and PVN of the thalamus. Cells exhibiting moderate to weak ERβ-ir were observed in several regions of the isocortex (see Table 1); striatum; ventral pallidum; subiculum; CA3 region of the hippocampus; internal capsule; substantia innominata; nucleus accumbens; cortical, amygdaloid, and central nuclei of the amygdala; several thalamic nuclei (central medial, rhomboid, and habenular); and the subincertal nucleus. Similar levels of nuclear ERβ-ir were observed in cells of the anterior and lateral hypothalamic areas; the arcuate, periventricular, supraoptic, and suprachiasmatic nuclei; the tuber cinereum area; and the ventrolateral division of the ventromedial nucleus. Interestingly, cells within the most caudal extent of the arcuate and ventromedial nuclei exhibited darker nuclear ERβ-ir compared with the rest of these nuclei, in which low to moderate labeling was seen (Fig. 4; levels, –1.34 and –1.70).

In most brain areas ERβ-ir was observed distinctly within the nuclear compartment of positive cells. Such nuclear-ir ranged from extremely dense, punctate stain, as seen in the medial amygdala and dorsal raphe (Fig. 5, B and D), to weak nuclear-ir, as observed in scattered cells within the CA3 pyramidal layer of the hippocampus (Fig. 3F). In the CA3 stratum lucidum, cytoplasmic labeling was observed in cross-sectional fibers (Fig. 3F). In the olfactory bulb, while nuclear-ir was seen within small cells of the glomerular layer, distinct cytoplasmic fiber labeling were observed in the external plexiform layer (Fig. 5A). Less intense fiber labeling was seen through the CA1 stratum radiatum of the hippocampus (data not shown).

Midbrain/pons. Regions that exhibited the most abundant nuclear ERβ-ir included the reticular division of the substantia nigra (caudal), the ventral tegmental area, the midbrain raphe nuclei (caudal linear, dorsal, and median), the pedunculopontine tegmental nucleus, and the locus coeruleus. Representative images of intense nuclear ERβ-ir are depicted in Fig. 3 (locus coeruleus) and Fig. 5 (substantia nigra and dorsal raphe nucleus). Areas observed to have moderate levels of ERβ-ir included the dorsal terminal and Edinger-Westphal nuclei; the compact and lateral divisions of the substantia nigra (caudal); the parabrachial pigmented nucleus; the rostral linear raphe; B9 serotonin cells; pontine and

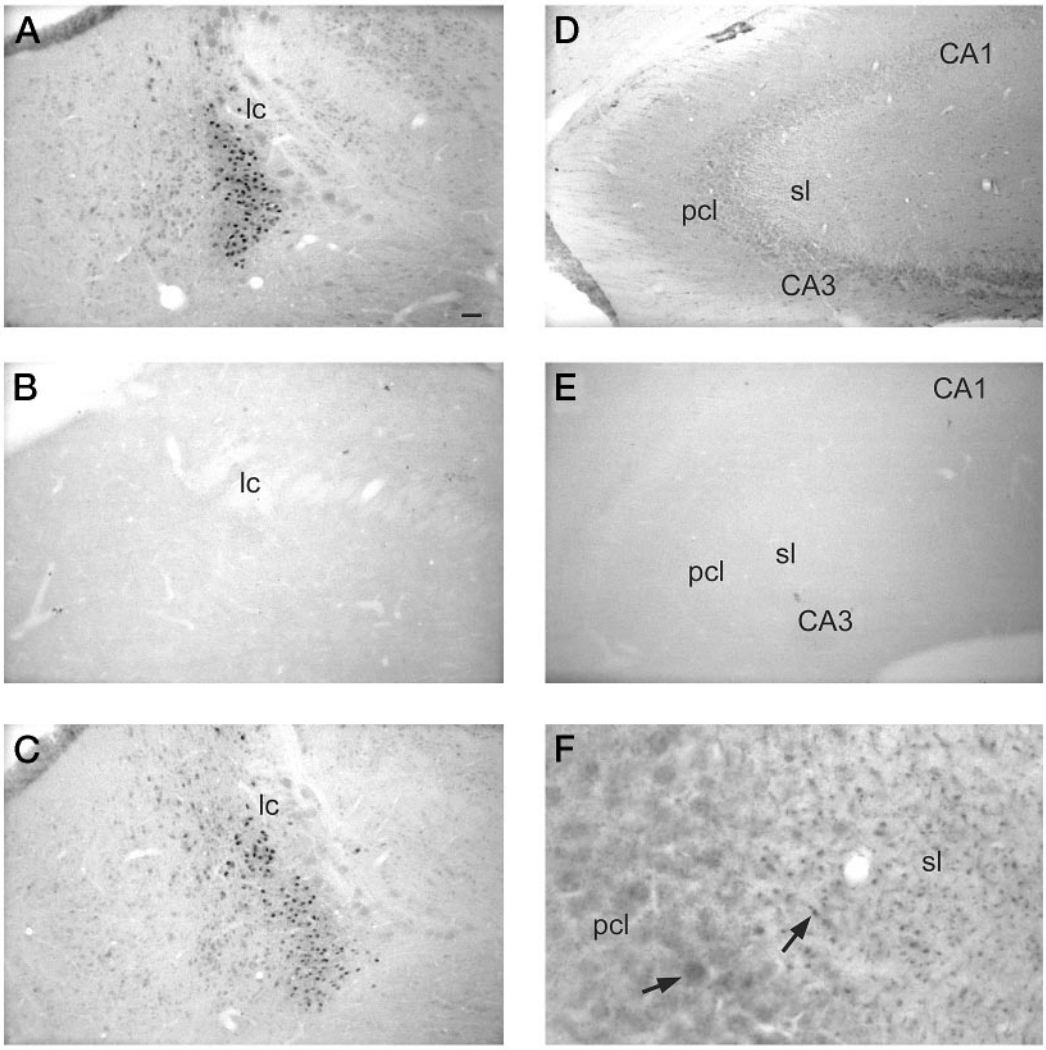

FIG. 3. Demonstration of the specificity of 80424 to ERβ in mouse brain. Nuclear ERβ-ir in the locus coeruleus (lc): A, control; B, preabsorption with the immunogenic peptide; and C, preabsorption with a nonspecific peptide within the ERβ sequence. Extranuclear-ir in hippocampus: D, control; E, preabsorption with the antigenic peptide; and F, a higher magnification of cross-sectional fibers in the CA3 stratum lucidum (*right arrow*). Note the moderate nuclear label in select CA3 pyramidal cells (*left arrow*). lc, Locus coeruleus; pcl, pyramidal cell layer; sl, stratum lucidum. *Size bar*: A–E, 50 μm; F, 10 μm.

pontine reticular nuclei; subbrachial and paralemniscal nuclei; the lateral lemniscus; the parabigeminal, laterodorsal tegmental, reticulotegmental, and anterior tegmental nuclei; the medioventral periolivary nucleus; and the periolivary nuclei. Some nuclear ERβ-ir also was seen in the deep mesencephalic, interfascicular, and the lateral and medial vestibular nuclei. Interestingly, although distinct fiber labeling was seen in the dorsal medial division of the periaqueductal gray above the central aqueduct, little or no somal or nuclear ERβ-ir was observed in this brain region.

Hindbrain/cerebellum. Cells exhibiting strong nuclear ERβ-ir were recorded in the medullary raphe nuclei (magnus, pallidus, and obscurus) and the dorsal motor 10 nucleus of vagus. Moderate ERβ-ir levels were observed in the parapyramidal, gigantocellular reticular (α), rostroventrolateral reticular inferior olive (principle nucleus), lateral reticular nucleus, and spinal 5 nucleus (oral). In the cerebellum, weak to moderate nuclear ERβ-ir was observed in the pyramidal cell layer and also scattered within granule cells (Fig. 5F). Fiber/somal label was also evident in the cerebellum. In the remaining hindbrain, scattered cells exhibiting immunolabel were seen in the supratrigeminal nucleus, intermediate reticular nucleus, prepositus nucleus, and the nucleus ambiguous. Somal labeling was observed within cells of the facial nucleus.

Dark nuclear label also was identified within cells interspersed within areas of white matter, including the facial nerve and the cerebellar peduncle. These cells could be infiltrated neurons scattered within the myelinated tracts and/or glial cells such as oligodendrocytes.

ERα localization in brain using AS409 antibody

The general distribution patterns of nuclear ERα-ir and ERβ-ir in the mouse brain were similar, although there were differences in the abundance of receptor labeling in many overlapping regions as well as several areas that exhibited

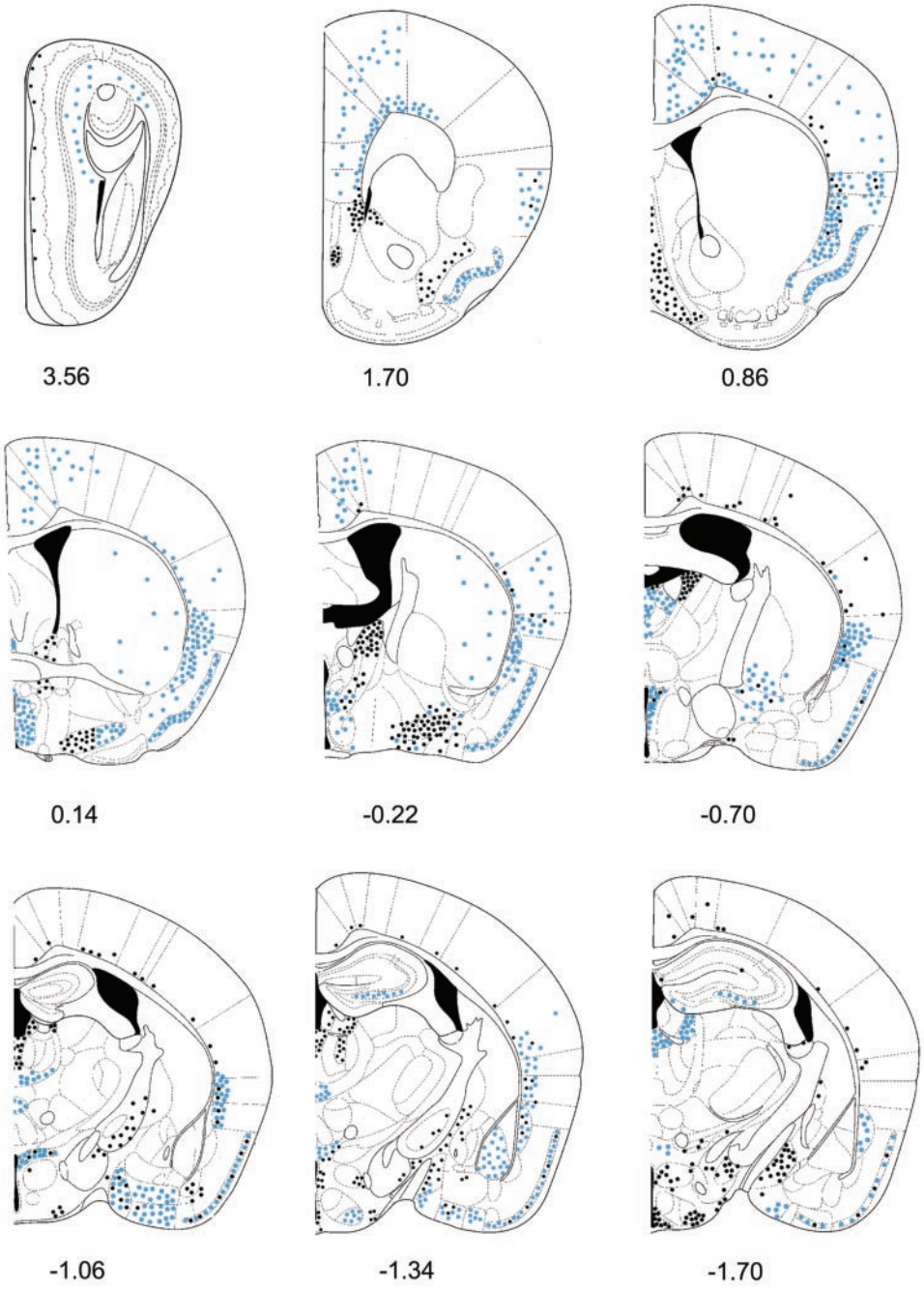

FIG. 4. The distribution of cells exhibiting nuclear ERβ-ir in the brain of an ovariectomized adult mouse. *Black dots* represent darkly labeled immunoreactive cells; *blue dots* represent cells with low moderate immunolabeling. Maps were drawn from actual 30-μm sections from two representative animals. The brain levels depicted are measured as distances from bregma (B) and are modified from a mouse brain atlas (42).

distinct expression of one receptor isoform. The selectivity of AS409 and 80424 antibodies to distinguish ERα or ERβ, respectively, in mouse brain, is demonstrated in Fig. 6. The AS409 antibody specifically recognized cells in the lateral septum that appeared to exclusively express ERα, whereas 80424 detected cells that express only ERβ within the medial septum and vertical limb of the diagonal band.

Forebrain. Forebrain regions that were observed to exhibit the most abundant nuclear ERα-ir included the BNST, medial amygdala, medial preoptic nucleus, arcuate nucleus, and ventrolateral division of the ventromedial nucleus of the hypothalamus. Other areas with pronounced nuclear ERα-ir included the islands of Calleja, lateral septum, septohippocampal nucleus, ventral hippocampus, amygdalohippocampal nucleus, anteroventral periventricular nucleus, medial preoptic area, dorsomedial and periventricular nuclei of the hypothalamus, and the central division of the ventromedial nucleus. Scattered cells exhibiting nuclear ERα-ir were recorded in the granule cell layer of the olfactory bulb; several cortical areas (granular insular, infralimbic, perirhinal, and second somatosensory cortexes); striatum (caudate putamen); ventral pallidum; subfornical organ; dorsal hippocampus; internal capsule; substantia innominata; anterior, basal, and central amygdaloid nuclei; and the precommisural, pretectal, and lateral posterior thalamic nu-

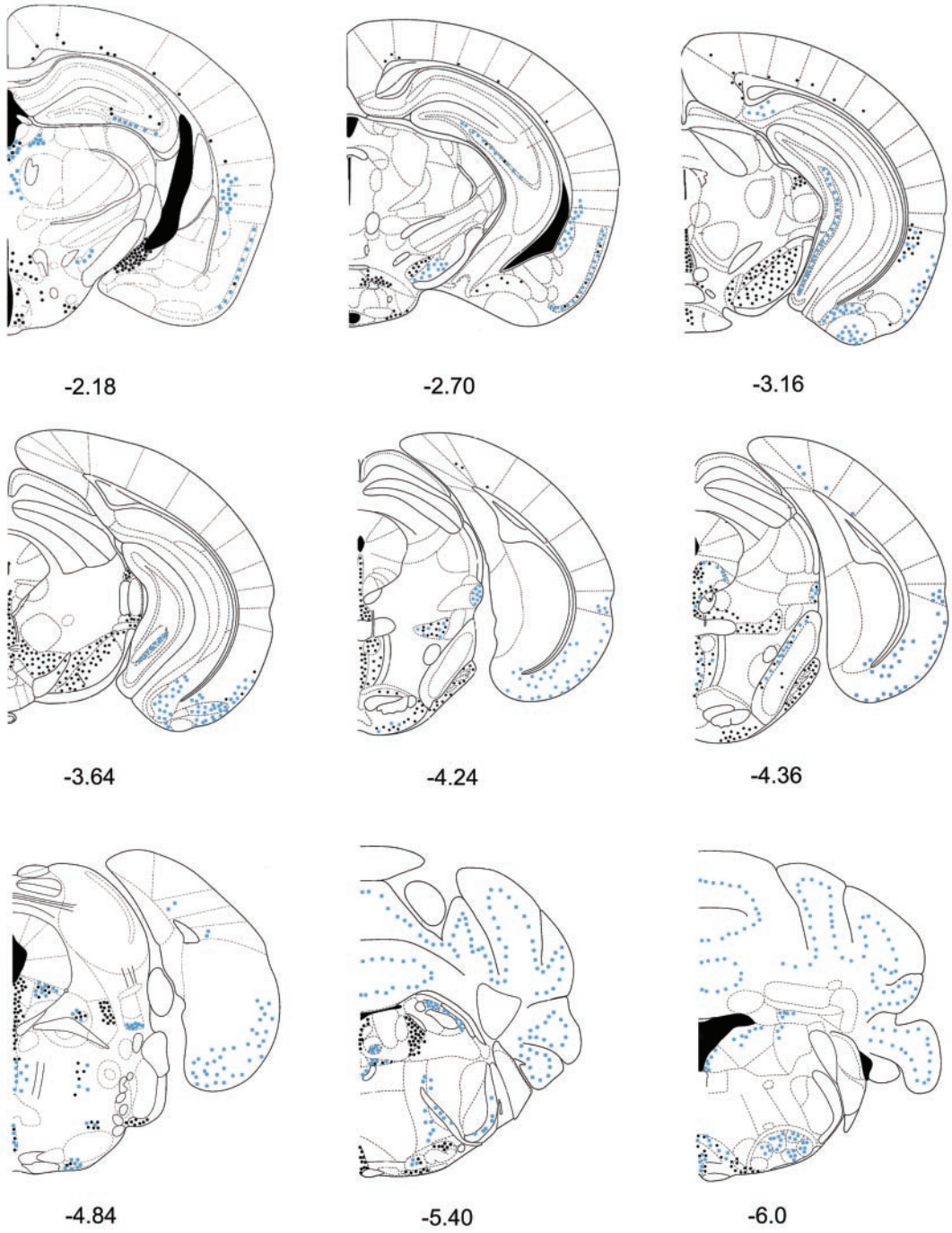

FIG. 4. *Continued.*

clei. In the preoptic area-hypothalamus, scattered nuclear ERα-ir was seen in the lateral and magnocellular preoptic areas, anterior commissural nucleus, anterior hypothalamic area, lateral hypothalamic area, medial tuberal nucleus, PVN, posterior hypothalamic area, supraoptic nucleus, tuber cinereum area, dorsomedial ventromedial nucleus, and across the mammillary nuclei.

Midbrain/pons. The periaqueductal gray and the lateral parabrachial nucleus exhibited the most abundant nuclear ERα-ir at this brain level. Other regions of the midbrain/pons that exhibited moderate to low levels of ERα-ir included the deep mesencephalic, dorsal terminal, and interfascicular nuclei; compact subdivision of the substantia nigra; inferior colliculus; midbrain raphe nuclei; pontine reticular, intercollicular, subbrachial, and laterodorsal tegmental nuclei; trapezoid body; locus coeruleus; medial parabrachial nucleus; and dorsal periolivary region. Some ERα-ir also was recorded in the interpeduncular nucleus; lateral division of the substantia nigra; superior colliculus; nucleus brachium inferior colliculus; B9 serotonin cells; pedunculopontine tegmental, medioventral periolivary, subcoeruleus, and intermediate reticular nuclei; periolivary nuclei; and prepositus nucleus.

TABLE 1. Distribution of ERα and ERβ immunoreactivity in the adult ovariectomized mouse brain

	ERα	ERβ
FOREBRAIN		
Olfactory bulb		
External plexiform layer	−	Fibers
Glomerular layer	−	+
Granule layer	−/+	+
Islands of Calleja	++	−
Cortex		
Allocortex		
Piriform	−	+
Dorsal endopiriform nu	−	+
Isocortex		
Agranular insular	−/+	+
Cingulate	−	−/+
Dorsal peduncular	−	−/+
Granular insular	−/+	+
Infra-limbic	−/+	+
Prelimbic	−	+
Motor	−	−/+
Somatosensory	−/+	−/+
Perirhinal	−/+	+
Retrosplenial agranular	−	−/+
Retrosplenial granular	−	−/+
Visual	−	−/+
Dorsal tenia tecta	−/+	++
Claustrum	−	++
Striatum		
Caudate putamen	+	−/+
Lateral striatal stripe	−	+
Globus pallidus	−	++
Septal complex		
Lateral nu	++	−
Medial nu	−	+
Septohippocampal nu	++	+
Diagonal band of Broca		
Horiz limb diagonal band	−/+	+++
Vert limb diagonal band	−/+	+++
Accumbens nu		
Shell	−/+	−
Core	+	−/+
Ventral pallidum	+	−/+
Subfornical organ	+	−
Hippocampal formation–dorsal		
CA1	+	Fibers
CA2	−/+	−
CA3	+	−/+
Stratum lucidem	+	Fibers
Dentate gyrus hilus	+	−/+
Subiculum	−	+
Hippocampal formation–ventral		
CA1–CA3	++	−/+
Entorhinal cortex	−/+	+
Internal capsule	−	+
Bed nucleus of the stria terminalis	+++	+++
Interstial nu post limb ant commisure	+	−
Olfactory tubercle	+	−
Substantia innominata	+	+
Amygdala		
Anterior amygdaloid area	+	+
Amygdalohippocampal nu	++	+
Basolateral nu	−/+	+
Basomedial nu	+	+
Cortical amygdaloid nu	+	−/+
Central nu	+	−/+
Medial nu	+++	+++
Thalamus		
Anterodorsal nu	−	+
Mediodorsal nu	−	+
Central medial nu	−	+
Paraventricular nu	−	++
Rhomboid nu	−	+
Habenular nu	−/+	+
Precommisural nu	+	−/+
Pretectal nu	+	−
Lateral posterior thalamic nu	+	−
Zona incerta	+	−
Subincertal nu	+	−/+
Preoptic area		
Anterovent periventricular nu	++	+
Lateral preoptic area	+	−/+
Medial preoptic area	++	−/+
Magnocellular preoptic area	−/+	++
Medial preoptic nu	+++	+
Hypothalamus		
Anterior commissural nu	−/+	−
Anterior hypothalamic area	+	−/+
Arcuate nu	+++	+
Dorsomedial nu	++	++
Lateral hypothalamic area	+	−/+
Medial tuberal nu	+	++
Paraventricular hypothalamic nu		
Magnocellular divisions	+	++
Parvicellular divisions	+	++
Periventricular hypothalamic nu	++	+
Posterior hypothalamic area	+	+
Subthalamic nu	−	−/+
Suprachiasmatic nu	−	−/+
Supraoptic nu	−/+	−/+
Tuber cinereum area	+	+
Ventromedial hypothalamic nu		
Dorsomedial	−/+	−
Central	++	−
Ventrolateral	+++	+
Lateral mammilary nu	+	−
Medial mammilary nu	−/+	+
Supramammillary nu	+	++
Ventral tuberomammilary nu	+	+
MIDBRAIN/PONS		
Deep mesencephalic nu	+	−/+
Dorsal terminal nu	+	+
Edinger-Westphal nu	−	+
Interfascicular nu	+	−/+
Interpeduncular nu	−/+	−/+
Suprageniculate thalamic nu	−/+	−
Substantia nigra (caudal)		
Compact	+	++
Lateral	−/+	+
Reticular	−	+++
Ventral tegmental area	−	+++
Nu brachium inferior colliculus	−/+	−
Parabrachial pigmented nu	−	+
Subbrachial nu	+	+
Periaqueductal gray	+++	−/+
Dorsal medial	++	Fibers
Superior colliculus	−/+	−
Inferior colliculus (external ctx, 2)	+	−
Raphe nuclei		
Rostral linear	−	+
Caudal linear	+	++
Median/paramedian	+	++
Dorsal raphe	+	+++
B9 serotonin cells	−/+	+
Pontine nuclei	−	+
Pontine reticular nu		
Oral	−/+	+
Caudal	−/+	−/+
Intercollicular nu	+	−
Paralemniscal nu	−	+

TABLE 1. *Continued*

	ERα	ERβ
Lateral lemniscus		
Intermed nu	−	+
Ventral nu	−	−/+
Parabigeminal nu	−/+	+
Laterodorsal tegmental nu	−/+	+
Pedunculopontine tegmental nu	−/+	++
Reticulotegmental nu pons	−	+
Anterior tegmental nu	−	+
Superior Olive		
Medioventral periolivary nu	−	+
Rostral periolivary region	+	−/+
Dorsal periolivary region	−	+
Superior paraolivary nu	−	+
Caudal periolivary nu	−/+	−/+
Nu of trapezoid body	+	−/+
Locus coeruleus	+	+++
Subcoeruleus nu	−/+	+
Parabrachial		
Medial nu	+	−
Lateral nu	++	+
Sensory root trigeminal nerve	−	+
Spinal trigeminal tract	−	+
Medial vestibular nu	−	−/+
Lateral vestibular nu	−	−/+
HINDBRAIN/CEREBELLUM		
Raphe nuclei		
Magnus	−	++
Pallidus	−	++
Obscurus	−	+
Middle cerebellar peduncle	−	+
Cerebellum	−	+
Posterodorsal tegmental nu	+	+
Supratrigeminal nucleus	+	−/+
Facial nerve	−	+
Facial 7 nu	−	Cytoplasmic
Parapyramidal nu	+	+
Intermediate reticular nu	−/+	−/+
Gigantocellular reticular nu, alpha	−	+
Gigantocellular reticular nu	+	−
Lateral reticular nu	+	+
Paragigantocellular reticular nu	+	−
Rostroventrolateral reticular nu	+	+
Nu ambiguous	−	−/+
Prepositus nu	−/+	−/+
Nu solitary tract	++	−
Area postrema	+	Fibers
Inferior olive, principal nu	−	+
Dorsal motor 10 nu of vagus	−/+	++
Spinal 5 nu, oral	−/+	+
Dorsomedial spinal 5 nu	+	−
Spinal 5 nu	+	−

nu, Nucleus.

Hindbrain/cerebellum. The nucleus of the solitary tract exhibited the most robust nuclear ERα-ir in the hindbrain. Other areas with distinct ERα-ir included the posterodorsal tegmental nucleus; supratrigemenal, parapyramidal, gigantocellular and paragigantocellular reticular nuclei; area postrema; rostroventrolateral reticular and lateral reticular nuclei; and dorsomedial spinal 5 and spinal 5 nuclei. Further, some nuclear ERα-ir was seen in the intermediate reticular and prepositus nuclei, dorsal motor 10 nucleus of vagus, and the spinal 5 nucleus, oral division. No distinct ERα-ir was observed in the cerebellum.

As was seen for ERβ-ir, the majority of ERα-ir was distinctly within cell nuclei. Across various regions of the brain, some somal immunostaining could be seen as a halo around positively labeled nuclei (data not shown). In general, there was far less distinct extranuclear ERα-ir, such as fiber labeling, compared with what was seen for ERβ-ir in select brain regions. However, ultrastructural evidence indicates that this receptor can be found in discrete extranuclear sites (dendritic spines, axons, and axon terminals) in the rodent brain (5, 8).

Discussion

In the present study we describe the characterization of an ERβ antibody and its use, along with a previously characterized ERα antibody, to document the localization of these ER subtypes in the brain of the ovariectomized mouse. This work represents the most comprehensive description of ER distribution specifically in the murine CNS. Although the distribution patterns of both receptor subtypes are generally similar, several brain regions exhibit a preferential expression of one isoform, and some regions appear to express only one ER subtype. Data from select brain regions will be highlighted below, and our findings will be compared with previously reported data in the rat and mouse.

Specificity of the 80424 antibody

The novel polyclonal ERβ peptide antibody 80424 selectively recognizes ERβ in mouse, rat, and human tissue, as determined by immunoblot analysis and immunocytochemistry in transfected cells and in mouse brain sections. The apparent size of the approximately 70-kDa band detected in rodent brain and human testicular extracts appears to be larger than the expected size of cloned ERβ and is suggestive of possible posttranslational modifications, including acetylation, phosphorylation, and/or glycosylation. Further, the major band seen in extracts of the human ovary (~55 kDa) and the second band seen in the testicular extracts were slightly smaller than the predicted size (~60 kDa) of recombinant human ERβ, perhaps as a result of posttranslational modifications. Immunocytochemical staining in transfected COS-7 cells was localized specifically to cell nuclei.

A number of ERβ isoforms have been reported in most or all species examined (43–47). In the mouse several isoforms have been identified with altered ligand-binding domains, deletions of exon 5 or 6 or insertion of an additional 54 nucleotides between exons 5 and 6 (46, 47), thus significantly reducing estrogen binding. The 80424 antibody does not distinguish between these alternate isoforms, as the epitope spans exons 2 and 3. Thus, the distribution of ERβ-ir that we are reporting in the mouse brain presumably represents both high and low affinity (or nonbinding) receptors. Therefore, this distribution may not completely overlap with estrogen binding data in the mouse brain.

Comparison of ER subtype distribution in the mouse brain

A comparison of ERα-ir and ERβ-ir in the brain of the adult ovariectomized mouse provides insight into the role each ER subtype may play in murine brain function. However, as ER

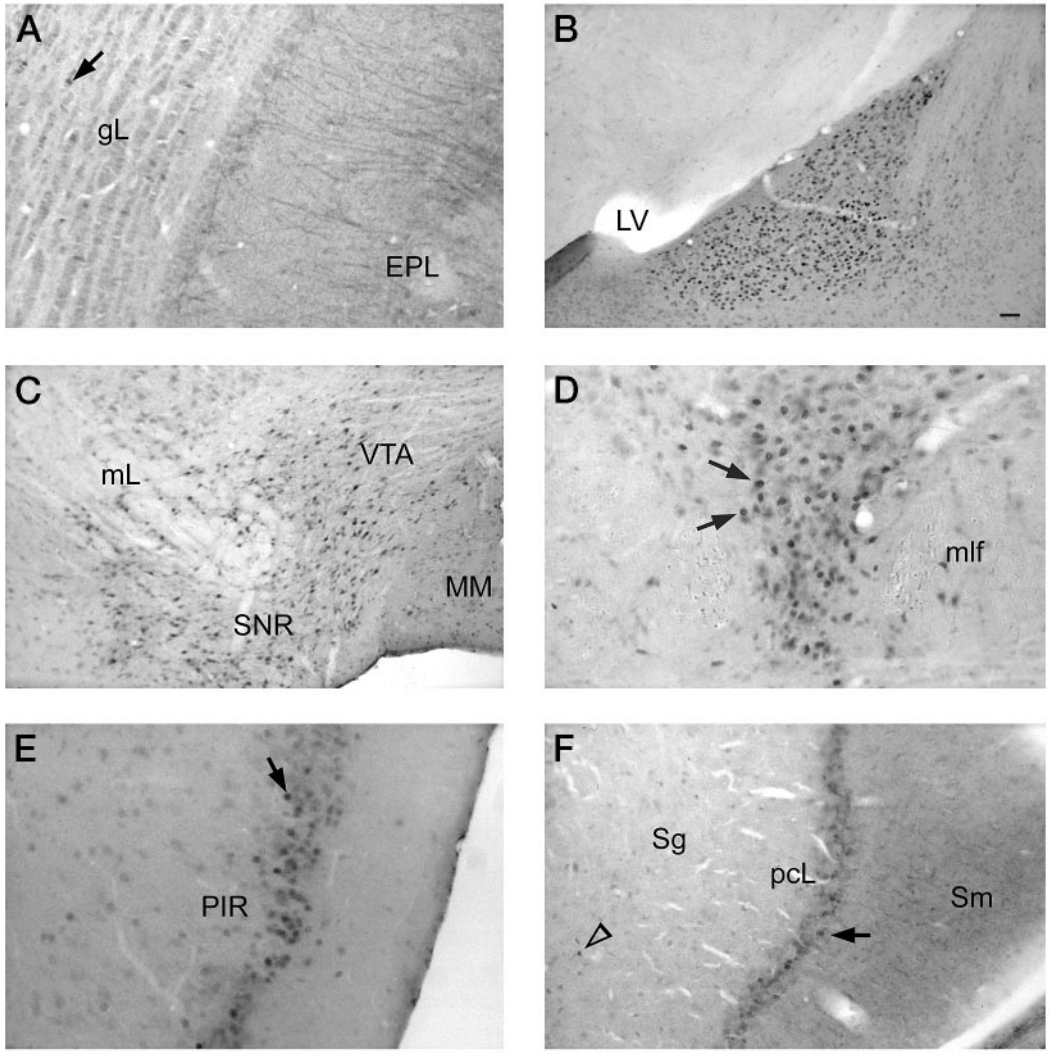

FIG. 5. ERβ-ir as determined by the 80424 antibody in select regions of the mouse brain. A, Nuclear (*arrow*) and extranuclear (fibers in the external plexiform layer) labeling is seen in the olfactory bulb, whereas nuclear labeling is seen in the medial amygdala (B), substantia nigra/ventral tegmental area (C), dorsal raphe (D), piriform cortex (E), and cerebellum, stratum granulosum (*arrowhead*) and pyramidal cell layer (*arrow*) (F). EPL, External plexiform layer; gL, glomerular layer; LV, lateral ventricle; mL, medial lemniscus; mlf, medial longitudinal fasiculus; MM, medial mammillary nucleus; pcL, pyramidal cell layer; PIR, piriform cortex; Sg, stratum granulosum; Sm, stratum moleculare; VTA, ventral tegmental area. *Size bar*: B, C, and F, 50 μm; A, D, and E, 25 μm.

expression patterns are likely to vary in the developing, intact cycling adult and/or aging female brain, these data should be considered to represent ER distribution specifically in the estrogen-depleted young adult murine brain. Although the expression of ERα is estrogen regulated (39), less is known regarding estrogen modulation of ERβ expression in the CNS. For example, although estrogen replacement did not diminish ERβ mRNA in brain regions examined in the spayed macaque (26), some rodent studies have reported estrogen regulation of ERβ mRNA or protein in a region-specific manner (23, 48–50).

In the forebrain, areas such as the BNST and the caudal extent of the medial amygdala exhibited nearly equally abundant nuclear-ir to ERα and ERβ. Although robust ERα-ir was found throughout the extent of the medial amygdala, ERβ-ir cells were most heavily concentrated caudally. The abundance of both ERs in the caudal aspect of the medial amygdala suggests that the two receptors may be expressed in the same cells and perhaps form functional heterodimers, as has been demonstrated *in vitro* (51, 52). Both ER subtypes have been reported to colocalize in the rat medial amygdala (48, 53) as well as the anteroventral periventricular nucleus of the preoptic area (18, 48).

Throughout the cerebral cortex, ERβ was observed to be the predominant receptor subtype; most cells exhibited light to moderate nuclear-ir. However, there were scattered cells with darkly labeled nuclei, particularly those found adjacent to the external capsule and cingulum and scattered in the piriform cortex. The ERβ also has been reported to be the predominant ER subtype in the cerebral cortex of the rat (14); however, the expression pattern differs greatly between these two rodent species. In the rat there is a more extensive and consistent expression pattern of strong nuclear label throughout laminae IV and VI (13), whereas in the mouse

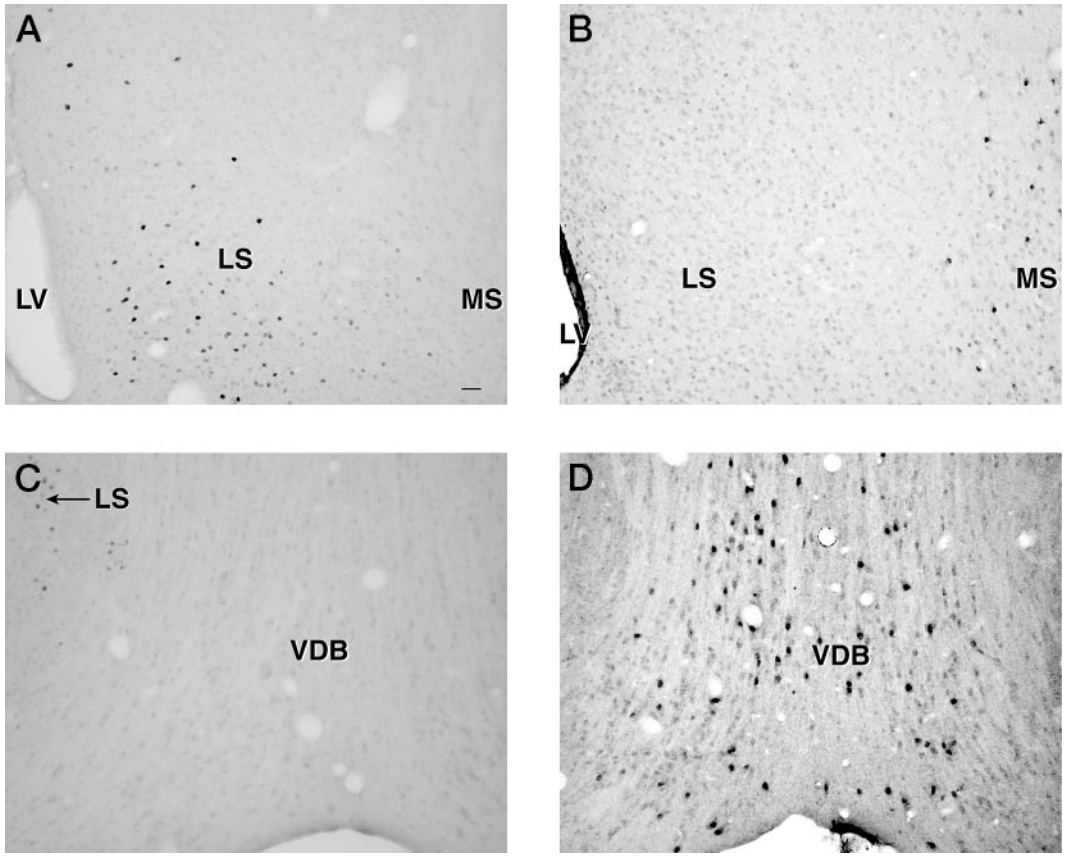

FIG. 6. A comparative distribution of nuclear ERα-ir (A and C) and ERβ-ir (B and D) in the septal complex. Note the distribution of cells exhibiting ERα-ir specifically in the lateral septum (A and C), whereas distinct ERβ-ir is found in cells exclusively in the medial septum (B). Ventrally, robust ERβ-ir is found in the vertical limb of the diagonal band (D), whereas ERα is essentially absent from this forebrain region (C). These findings clearly demonstrate the selectivity of the ERα and ERβ antisera used in the present study. LS, Lateral septum; LV, lateral ventricle; MS, medial septum; VDB, vertical limb of the diagonal band. *Size bar*, 50 μm.

cortex, immunolabeling is found in select subregions (*i.e.* somatosensory and insular regions) and is predominantly low to moderate in intensity.

The basal forebrain exhibited both ER subtypes in varying levels. In the striatum, scattered cells exhibited light to moderate nuclear-ir for both receptors, as previously reported in a developmental study of the mouse striatum (25), but the ERα-ir was generally more intense. Moderate to intense nuclear ERβ-ir was observed in cells within the globus pallidus, whereas little ERα-ir was found in this brain region. Within the septum, ERα was found specifically within cells of the lateral nucleus, whereas ERβ-ir was observed almost exclusively within cells in the medial nucleus, a largely cholinergic region. Both the horizontal and vertical limbs of the diagonal band of Broca, other major cholinergic areas, contained many cells with intense ERβ-ir, whereas little ERα-ir was observed in these regions. Once again the mouse appears to differ from the rat, as these basal forebrain regions in the rat express both ER subtypes in abundance (14), and nearly all of the ER-expressing cholinergic neurons in the rat have been reported to exhibit specifically ERα-ir (54).

Both receptor subtypes were observed in the preoptic area and hypothalamus, with regional variations. ERα predominated in the medial preoptic area and the arcuate and ventromedial nuclei of the hypothalamus, regions important in the regulation of reproductive physiology and behavior. These findings are consistent with the profound reproductive abnormalities seen specifically in the αERKO mouse (2, 20, 55–57). The ERβ was more abundant in the magnocellular preoptic area and the PVN. The rat PVN was first identified as a major site of ERβ expression (14–17). Although the rat PVN has been reported to express only ERβ (14), we have found that the murine PVN contains cells that express moderate levels of ERα. Thus, both ER subtypes may contribute to estrogen's actions in the mouse PVN. Interestingly, the human PVN also has been reported to express mRNA for both ERα and ERβ; in fact, ERα appears to be the predominant subtype in the human PVN (27).

Within the midbrain, pons, and brainstem, a predominance of ERβ was observed in several of the major monoaminergic cell populations. These include the heavily dopaminergic substantia nigra and ventral tegmental area, the serotonergic raphe nuclei, and the noradrenergic locus coeruleus. Although light to moderate ERα-ir also was observed in some of these regions, the number and intensity of cells exhibiting nuclear ERβ-ir were far more abundant. These findings suggest that estrogen is likely to regulate these monoaminergic systems primarily through the ERβ. Regarding the dorsal raphe nucleus, this appears to be con-

sistent across species examined, specifically the mouse (40), rat (14, 58), macaque (26), and guinea pig (59). The cerebellum of the adult mouse appears to be similar to that of the adult rat (14), as only ERβ-ir was seen in this brain region, although the intensity of immunolabel appeared to be generally weaker in the mouse compared with what has been reported in the rat (15).

Our data for ERβ-ir in mouse brain are generally in agreement with a previous report describing the distribution of ERβ mRNA in the forebrain and rostral midbrain of the αERKO mouse (24). Notable exceptions include the thalamus, substantia nigra, and ventral tegmental area. We observed cells with moderate to robust nuclear ERβ-ir within several thalamic nuclei and robust nuclear ERβ-ir in the substantia nigra and ventral tegmental area, yet no hybridization signal for ERβ was reported in these regions in the αERKO mouse (24). Further, although we observed only weak ERβ-ir in the suprachiasmatic nucleus, this region was reported to express abundant mRNA. Considering that we examined wild-type mice in the present study, it is conceivable that disruption of the ERα gene could result in differences in ERβ expression in select regions. Indeed, very recent evidence indicates that ERβ expression is greatly modified in specific brain regions in the male αERKO mouse compared with wild-type siblings (50). Alternatively, these areas may express levels of mRNA that are not proportional to the transcribed protein.

Regarding extranuclear immunostaining, several areas of the mouse brain exhibited distinct fiber labeling for ERβ, most notably in fibers of the olfactory bulb and in cross-sectional fibers of the CA3 stratum lucidum of the hippocampus. This labeling was absent upon preabsorption with the cognate peptide, suggesting that the signal was indeed specific for this ERβ sequence. Cytoplasmic distribution of ERβ-ir has been reported by several groups using other antibodies (16, 17, 60–62). In the present light microscopy study, extranuclear ERα-ir was less evident, mostly appearing as light somal immunostaining surrounding intense nuclear-ir. Despite little extranuclear ERα-ir evident at the light microscopic level, several studies examining the ultrastructural distribution of ERα using multiple antibodies have identified this receptor in discrete subcellular compartments in the rat brain. Specifically, such labeling has been reported in axons and axon terminals in the rat hypothalamus (63, 64) and more recently in axons, axon terminals, and dendritic spines in neurons of the hippocampus (5, 8). Further studies are currently underway to determine the subcellular distribution of ERβ-ir.

Conclusion

Our data demonstrate the extensive, but region-specific, distribution of ERβ and ERα in the mouse brain. This work represents the most comprehensive description of ER distribution in the murine CNS. The mouse has become an important animal model in neuroscience research based largely on the well characterized genome and the technology to generate knockout/transgenic lines in this rodent species. Integration of the present findings of ER subtype distribution into the established network of neuronal pathways provides an anatomical basis from which we may further our understanding of estrogen's mechanisms of action on murine brain function. Further, together with data reported in other species, these findings confirm that there are both similarities and differences in expression patterns of ERα and ERβ in the CNS between species.

Acknowledgments

We thank Dr. Ellen Bayne and Michael Dashkevicz for their technical assistance, and Dr. Teresa Milner (Weill Medical College of Cornell University) and Dr. Eva Mezey (NIMH, NIH) for her consultations in neuroanatomy.

Received October 15, 2002. Accepted January 10, 2003.

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This work was supported in part by NIH Grant NS-07080 (to B.S.M.), NIMH 62147 (to S.O.), and HD-05751 (to D.W.P.).

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