Regional, Laminar, and Cellular Distribution of Immunoreactivity for ERα and ERβ in the Cerebral Cortex of Hormonally Intact, Adult Male and Female Rats

Behavioral, biochemical and anatomical studies suggest that estrogen stimulates structure and/or function in the adult cerebral cortex. The studies presented here used immunocytochemistry to map the α and β isoforms of intracellular estrogen receptors (ERα, ERβ) in major subdivisions of adult rat cortex to identify potential sites for relevant receptor-mediated hormone actions. These studies revealed that immunoreactivity for ERα (ERα-IR) and ERβ (ERβ-IR) was present in most cortical areas, was associated exclusively with neurons, and was similar in males and females. Each receptor isoform also had its own unique distribution with respect to cortical regions, layers, and cells. In sensorimotor areas, for example, ERβ-IR was more prominent than ERα-IR, and was concentrated in layer V neurons that were immunoreactive for parvalbumin. In contrast, ERα-IR was scattered among parvalbumin-immunonegative cells in layers II/III and V/VI. Likewise, in entorhinal cortex, ERβ-IR was present in calbindin-containing cells in layers III–VI, while ERα-IR was restricted to small numbers of calbindin-negative neurons in infragranular layers. In sum, ERβ-IR and ERα-IR were differentially distributed both with respect to cortical compartments and with respect to each other. Accordingly, estrogen activation at these two sites may be anticipated to impact disparate sets of cortical circuits, cells, and functions.

Introduction
The cerebral cortex is a recognized target of ovarian hormone stimulation. In addition to developmental influences [e.g. see (Miranda and Toran-Allerand, 1992; Lustig et al., 1993; Stewart and Rajabi, 1994)], behavioral (Brower et al., 1981; Hampson, 1990), biochemical (Fink et al., 1996), and anatomical studies in humans and/or other mammals (Kritzer and Kohama, 1998, 1999; Kritzer, 2000) suggest that estrogen also stimulates the mature cerebrum. Among the mechanisms that could mediate this stimulation are intracellular receptor–hormone interactions. In the adult cortex in particular these could involve estrogen activation of the classical or α isoform of the ER (ERα) and/or the more recently identified β isoform of the ER (ERβ, see Kuiper et al., 1996), both of which seem to be present in the cortices of sexually mature animals (Li et al., 1997; Shughrue et al., 1997; Weiland et al., 1997; Mufson et al., 1999). There are hints, however, that the functional roles of ERα vs ERβ-mediated processes in the adult cerebrum may be very different. Both immunocytochemical and in situ hybridization studies, for example, indicate that the two receptor isoforms differ substantially in their relative abundance (Simerly et al., 1990; Li et al., 1997; Shughrue et al., 1997; Mufson et al., 1999), and in the cortical compartments within which they are localized (Shughrue et al., 1997). Because ligand activation at ERα vs ERβ sites can differentially induce morphological endpoints (Patrone et al., 2000) and gene transcription at, for example, AP-1 sites (Paech et al., 1997), a precise knowledge of the cortical localization of each receptor subtype is needed to evolve a complete understanding of how estrogen impacts cortical function and form. Because ERα and ERβ subunits can also form heterodimers that are functionally, i.e. transcriptionally, distinct from both ERα and ERβ homodimers (Pettersson et al., 1997; Ogawa et al., 1998), it may be equally important to determine whether and where in the cortex these two signaling pathways intersect. The studies presented here addressed each of these issues by using immunocytochemistry to generate detailed maps of the distributions of ERα and ERβ proteins in the cerebral cortices of hormonally intact adult male and female rats. Specifically, patterns of immunoreactivity for the ERα and ERβ receptor isoforms were each defined in relation to cytoarchitectonically identified regions and layers of all major subdivisions of the rat cerebral cortex. In these areas as well, both receptor isoforms were charted in relation to individual cells identified by cytoplasmic markers including glial fibrillary acidic protein, neuron-specific enolase, and the calcium binding proteins parvalbumin (PV) and calbindin (CB). Together these analyses provided a comprehensive, fine-grained definition of the distributions of ERα and ERβ in the adult rat cortex. Consideration of the known functional specializations of the particular cortical regions, layers, and cells that immunoreactivity for each receptor isoform occupied suggests that ERα- and ERβ-mediated pathways may indeed be poised to influence different cells and circuits in the adult rat cerebral cortex.

Methods

Tissue
A total of 18 adult Sprague–Dawley rats (12 males, 6 females) were used. All animals were hormonally intact adults weighing between 200 and 400 g. Vaginal cytology smears were taken to track the estrous cycles of all females prior to sacrifice, and were also used to verify which animals were in proestrus (n = 3) and which were in diestrus (n = 3) at the time of euthanasia.

Tissue Preparation
Rats were deeply anesthetized with a mixture of ketamine and xylazine (90 and 10 mg/kg, respectively) delivered intramuscularly. After behavioral checks were made to ensure the disappearance of deep reflexes, animals were transcardially perfused with 100–200 ml of phosphate-buffered saline flush, followed by 500–1000 ml of one of the following fixatives: 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 (two males); 4% paraformaldehyde containing 15% saturated picric acid in 0.1 M PB, pH 7.4 (two males); 2% paraformaldehyde containing 15% saturated picric acid in 0.1 M PB, pH 7.4 (four males, six females); 1% paraformaldehyde in 0.1 M PB, pH 7.4 (one male); 1% paraformaldehyde containing 15% saturated picric acid in 0.1 M PB, pH 7.4 (one male). Following perfusion, brains were removed and placed in 0.1 M PB containing 10%, 20%, and finally 30% sucrose for cryoprotection. After brains had sunk in the most concentrated sucrose solution, they were rapidly frozen in powdered dry ice, secured to a freezing microtome stage, and serially sectioned (40 µm) from the anterior to the posterior pole.

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Single Label Immunohistochemistry

For studies using only one antibody (anti-ERα or anti-ERβ), sections were first rinsed in 0.1 M PB, pH 7.4, reacted with 1% H2O2 in PB for 45 min, rinsed again in 0.1 M PB, and were then incubated in 1% sodium borohydrate in PB (45 min). Sections were then rinsed in 50 mM tris buffered saline (TBS), pH 7.4, and placed in blocking solution [TBS containing 10% normal swine serum (NSS) for 2 h at room temperature]. Sections were then incubated in primary antiserum, diluted in TBS containing 1% NSS, for 3 days at 4°C. After this incubation, sections were rinsed in TBS and then placed in biotinylated secondary antibodies (2 h, room temperature, working dilution 1:100). Sections were again rinsed in TBS, and incubated in avidin–biotin-complexed horseradish peroxidase (ABC, Vector Labs, Burlingame, CA; 2 h, room temperature). After additional rinses in TBS and then in tris buffer (TB), pH 8.0, sections were reacted using 0.07% 3,3′-diaminobenzadine (DAB) and 0.06% nickel ammonium sulfate as chromagen.

Antidiyptic Specificity

Polyclonal antibodies were used that selectively recognize the α and β isoform of the intracellular estrogen receptor (α isoform (Upstate Biotechnology, Lake Placid, NY, lot no. 185889), β isoform (Zymed Laboratories, South San Francisco, CA, lot no. 01162952)). The specificity of both antibodies has been tested on Western blots, and neither was pre-absorbed prior to use [see also Shughrue and Merchenthaler (Shughrue and Merchenthaler, 2001)]. The anti-ERα was used at a working dilution of 1:6000, and the ERβ antibody was used at a dilution of 1:750. Monoclonal antibodies recognizing neuron-specific enolase (NSE) and glial fibrillary acidic protein (GFAP) were purchased from Chemicon International Inc. (Temecula, CA), and were used at working dilutions of 1:100 and 1:300, respectively. The oligodendrocyte-specific monoclonal antibody (RIP) that was used was developed by Dr Susan Hockfield (Yale University, New Haven, CT) and was obtained from the Developmental Studies Hybridoma Bank, a facility maintained by the Department of Biological Sciences, University of Iowa, Iowa City, IA, under contract NO1-HD-7-3265 from the National Institute of Child Health and Development (NICHD); this antibody was used at a working dilution of 1:3. Monoclonal antibodies recognizing parvalbumin (PV) or calbindin (CB) were purchased from Sigma Chemical Corp. (St Louis, MO); both were used at working dilutions of 1:1000.

Control Experiments

The immunohistochemical labeling protocols (single and double) described above were carried out on representative sections from male and female animals with the omission of primary or secondary antibodies.

Data Analysis

Sections of the rat cerebrum analyzed were taken at 400 µm intervals from the rostral to caudal poles. In most cases cytoarchitecture was defined according to Zilles and Wree, in counterstained sections (Zilles and Wree, 1985). Any areas that were defined by other criteria are so identified in the text.

Quantitative Analysis

Detailed quantitative assessments of labeling were carried out in all major cytoarchitectonic divisions of the rat cerebral cortex. In each area, assessments were made of the appearance, regional and laminar distribution, relative abundance and degree of colocalization of ERα-IR and ERβ-IR with NSE, GFAP, RIP, PV and CB.

Results

The studies presented here describe the distribution of estrogen receptor-immunoreactivity in identified regions, layers, and cells of all major cytoarchitectonic divisions of the iso- and allocortex in hormonally intact adult male rats, and in adult female rats that were in either in proestrous or diestrous at the time of euthanasia. In no cases were obvious sex differences or estrus cycle-dependent variations in the appearance, distribution, or apparent density of receptor immunoreactivity observed. Accordingly, all of the data presented should be assumed to apply equally to all three animal groups evaluated.

Antibody Specificity

Commercially available antibodies were used in single- and double-label immunocytochemical studies to localize and colocalize the α and β isoforms of the intracellular estrogen receptor (ERα, ERβ). In sections labeled with the anti-estrogen receptor antibodies alone, immunoreactive (IR) profiles corresponded to small spots of blue/black DAB/nickel reaction product that were localized mainly to cell nuclei (Fig. 1). The gross distributions of these nuclei in the cerebral and cerebellar cortices, as well as in subcortical structures such as the amygdala and hypothalamus were good matches for previous descriptions of receptor proteins mapped using immunocytochemistry (Li et al., 1997; Weiland et al., 1997; Mufson et al., 1999), and/or for receptor mRNAs mapped using in situ hybridization (Simerly et al., 1990; Shughrue et al., 1997). For example, ERα-IR and ERβ-IR cells were both prominent in medial aspects of the amygdala, ERα-IR cells clearly outnumbered ERβ-IR cells in the peri-aqueductal gray, and ERβ-IR profiles predominated in the cerebellar and cerebral cortices. Immunolabeling for the five cytoplasmic markers used also gave rise to expected patterns of staining, with labeled cortical cells displaying morphologies typical of oligodendrocytes (anti-GFAP, Fig. 2B), astrocytes (anti-GFAP, Fig. 2A,C), or neurons (anti-NSE, anti-PV, anti-CB, see Fig. 2). No patterned or cellularly specific labeling was evident,
however, in control experiments in which the primary or secondary antibodies were omitted from immunolabeling protocols, further supporting the specificity of the antibodies used for marking intended antigens.

Sensitivity of ER-IR to Fixation

A number of earlier studies using immunocytochemistry to examine the distributions of ERα or ERβ in rat brain used animals that were perfused with fixatives containing acrolein [e.g. Weiland et al. and Jakab et al. (Weiland et al., 1997; Jakab et al., 2001)]. Initial experiments of this study tested whether appropriate signal-to-noise ratios of ER-IR could be obtained using alternatives to this fixative. The fixatives tested included paraformaldehyde solutions of varying strengths, as well as paraformaldehyde fixatives augmented with 15% saturated picric acid. These comparative studies revealed sensitivity of the anti-ERβ antibodies to conditions of fixation. Whereas immunolabeling for all of the other antibodies used were similar under all fixation regimens, immunoreactivity for ERβ (ERβ-IR) was markedly attenuated in brains fixed with 4% paraformaldehyde (with or without picric acid), and was considerably stronger in the more dilute aldehyde fixes. Among these, fixes combining

Figure 1. Representative high power photomicrographs showing immunoreactivity for the α and β isoforms of the intracellular estrogen receptor (ERα, ERβ) taken from layer V of the primary somatosensory cortex of a hormonally intact adult male rat. Immunoreactivity for both receptor types is concentrated over small nuclei that are usually round (A, B) but sometimes are more fusiform in shape (C, D). In some cases, patchy immunoreactivity for ERα can be seen extending into the cytoplasm and proximal processes of cell somata (A, D). Scale bar = 10 µm.

Figure 2. Representative color photomicrographs illustrating the anatomical relationships between nuclei immunoreactive for ERα or ERβ, and immunoreactivity for GFAP (A, C), the oligodendrocyte marker RIP (B), NSE (D–H), PV (I–L, N–O) or CB (K, M). Nuclei that were immunoreactive for ERβ or ERα were never found in cells expressing glial markers (A–C). Almost all receptor-positive nuclei were, however, sequestered within cells that were immunoreactive for NSE (D–H). Large and medium-sized pyramidal cells did not contain receptor-positive nuclei (e.g. D), but cells immunoreactive for ERα in deeper cortical layers did have vaguely pyramidal form (e.g. H). Cells that were immunoreactive for ERβNSE had bipolar (G), multipolar (E), or more irregular morphologies (F) that did not resemble pyramidal cells. Most of the ERβ-immunoreactive cells were also immunoreactive for PV (J, L, N). Neurons that were immunoreactive for ERα were almost always immunonegative for CB (M) and PV (O). Thus, in areas such as AID (N, O) where ERα and ERβ spatially overlapped, the two receptor isoforms appeared to be found in different cells populations. All sections shown are from hormonally intact adult male rats; unless otherwise indicated (N, O) sections are from primary motor or primary somatosensory cortex. Scale bars: K, M, N, O = 20 µm; all others = 10 µm.
the 15% picric acid solution with low paraformaldehyde concentrations (1 or 2%) consistently gave the strongest ERβ-immunoreactive signal along with almost undetectable levels of staining in surrounding tissue space. In choosing between the 1% paraformaldehyde–15% picric acid and the 2% paraformaldehyde–15% picric acid fixatives, which gave similar signal-to-noise ratios, the latter was selected as more strongly fixed tissue better withstand the protracted processing involved in double-label immunocytochemistry.

Identification of Cellular Phenotype of Cortical ER-IR Profiles

Both ERα-IR and ERβ-IR cells were distinguished by strong to moderate levels of immunolabeling that was concentrated over their nuclei (Fig. 1). Most labeled nuclei were round in shape and small to medium-sized (5–10 \(\mu\)m) in diameter (Fig. 1A,B). For both ERα and ERβ as well, occasional immunoreactive cells were also found that had more irregularly, e.g. fusiform, shaped nuclei (Fig. 1C,D). There were, however, subtle features of labeling that distinguished ERβ-IR from ERα-IR cells. Most notably, in ERβ-IR cells the DAB/nickel reaction product stood out sharply over nuclear compartments, but usually did not rise above background levels in the surrounding cytoplasm (Fig. 1B,C). In ERα-IR cells, on the other hand, labeled nuclei were often surrounded by cytoplasm that displayed weak, often patchy immunoreactivity that in some cases revealed enough morphology to suggest that the cells were neurons (Fig. 1A). For the most part, however, it was impossible to reliably classify cells as neuronal or glial based solely on labeling for ERα or ERβ alone. For this purpose, double-label immunocytochemistry experiments pairing ER-IR with immunoreactivity for NSE, GFAP, and the oligodendrocyte marker RIP were performed. These analyses revealed no ERβ-IR nuclei that were co-labeled with the glial markers (anti-GFAP or anti-RIP, Fig. 2A–C). However, nearly every ER-IR (α and β) nucleus encountered was surrounded by cytoplasm that was immunoreactive for NSE (Fig. 2D–H). For ERα-IR cells, the concomitant NSE labeling usually revealed neurons of two basic types. Nearer the pial surface, ERα-IR was typically found in neurons with NSE-labeled somata that were small and round and that emitted few obvious processes. Deeper within the cortex, however, ERα-IR was localized almost exclusively in small neurons with somata that were more triangularly shaped and that gave rise to single, striking apical processes (Fig. 2H). ERβ-IR, on the other hand, was localized in small NSE-IR cells that were most often either fusiform (Fig. 2F,G) multipolar, or more complex or irregular in shape (Fig. 2E). A final series of double-labeling experiments using antibodies recognizing the calcium binding proteins PV and CB revealed that only in exceptional cases were ERα-IR neurons also PV-IR or CB-IR. In contrast, the majority of the ERβ-IR neurons were PV-IR (Fig. 2J,L,N) and a few were CB-IR as well. As described separately by receptor subtype below, however, there was some degree of regional and laminar selectivity in the neurochemical signatures of cortical ER-IR cells.

Distribution of ERβ in the Cerebral Cortex

A comprehensive survey of the adult rat cerebral cortex revealed that only the transitional zone lying between the supragranular cingulate and granular retrosplenial cortices [see Van Eden and Uylings (Van Eden and Uylings, 1985)], was immunonegative for ERβ. In every animal examined this field alone stood out as uniquely lacking in receptor-bearing cells (Fig. 3). In all other major cytoarchitectonic division of the iso- and allocortex, modest to moderate complements of ERβ-IR cells were found. Further, for most regions, ERβ-IR was most prominent in layer V. The exceptions to this were the piriform and entorhinal cortices that are discussed in separate sections below.

ERβ in Sensorimotor and Association Areas of the Cerebral Cortex

The majority of sensory, motor, and association areas of the rat cerebrum displayed ERβ-IR that was most prominent in layer V. However, differences in the apparent density and/or in the laminar, tangential, or cellular distribution of receptor-bearing nuclei distinguished the association from the sensorimotor areas, and further distinguished some of the individual fields examined within both these two broad categories.

Association Areas: Regional and Laminar Distribution

The association areas of the cerebrum examined included cingulate, insular, and retrosplenial fields. Among these, the medial prefrontal (Cg1–3), retrosplenial (RSG and RSA), and ventral anterior insular (AIV) areas were each characterized by sparse complements of ERβ-IR cells that were distributed evenly and exclusively among the sublayers of lamina V (e.g. Fig. 4A–C). In the dorsal and posterior insular cortices (AID, AIP), receptor-

Figure 3. Low power photomicrograph showing a lack of immunoreactivity for ERβ in the transitional cortical zone as defined by Van Eden and Uylings (Van Eden and Uylings, 1985). Labeled nuclei are seen in layer V of the more medially located transitional zone. This area was unique among isocortical and allocortical areas alike in having no nuclei that were immunoreactive for ERβ in any of the animals examined. The tissue section shown is from a hormonally intact adult male rat. Abbreviation: cc = corpus callosum. Scale bar = 200 \(\mu\)m.
positive cells were also restricted to this same infragranular layer. However, in these regions ERβ-IR cells were much more numerous than in other association areas, and seemed to be more organized into clusters (compare Fig. 4A,B,C). Thus, in contrast to the scatter of ERβ-IR cells in, for example, area Cg1, receptor-bearing neurons in areas AID and AIP were often found arrayed in groups of three to five cells that overall had densities (per unit area) that were some three times higher than those of ERβ-IR cells in the cingulate areas.

Association Areas: Cellular Distribution
Double-labeling studies using receptor and calcium binding protein antibodies showed that, without exception, all of the ERβ-IR cells present in the association cortices were PV-IR (Fig. 4A–C). In most areas, these ERβ/PV-IR cells included equally prevalent examples of larger multipolar and smaller, morphologically simpler (e.g. bipolar) neurons. In areas AID and AIP, however, the ERβ/PV-IR cells corresponded almost exclusively to the larger, multipolar types of cells (Fig. 2N). Finally, all the association areas shared a modest complement of ERβ-IR cells lying mainly in the lower half of layer V that were also CB-IR (Fig. 5C). Because the ERβ-IR cells identified in alternate sections were all PV-IR, it was assumed that these ERβ/CB-IR cells represent subsets of receptor-bearing neurons that contained both calcium binding proteins.

Sensorimotor Areas: Regional and Laminar Distribution
The sensorimotor areas of the cortex examined included the
primary and secondary somatosensory (Par1, Par 2), auditory (Te1, Te3), visual (Oc1, Oc2), and motor (Fr1, Fr2) areas. Each of these regions shared a basic pattern of receptor immunoreactivity that was different in several respects from that seen in the association areas. First, although labeled cells were most conspicuous in layer V, the sensorimotor fields also had ERβ-IR cells located outside this layer. Thus, in every region examined there were moderate numbers of neurons seen in layer I, with smaller numbers of receptor-positive neurons also found in layers II/III and VI (Fig. 4A–F). The apparent density of ERβ-IR cells within layer V of the sensorimotor regions was also considerably higher than in the association areas (compare Figs 4B, C, and 4D–F). Quantitative estimates of the areal densities of ERβ-IR cells in representative areas of the frontal cortex bore this out. In the primary motor and somatosensory fields, for example, cell densities were on the order of three times higher than values for cingulate and insular areas.

Although immunoreactivity in all sensorimotor fields was similar, regional variations in labeling could also be appreciated. In many cases, these variations seemed to correspond to differences in the cytoarchitecture of layer V. For example, areas such as primary visual cortex where layer V is relatively compact had immunoreactivity that was focused into bands that were narrower than in, for example, motor areas where layer V is broader (Figs 4D–F). The somatosensory cortices, however, had more strikingly different patterns of immunolabeling. Specifically, whereas labeling in most areas of cortical zones was evenly distributed among the sublaminae of layer V, in areas Par1 and Par2 ERβ-IR was more restricted to the lower half of layer V, i.e. sublayer Vb (Fig. 6).

Sensorimotor Areas: Cellular Distribution
In addition to laminar differences, ERβ-IR in the sensorimotor versus association areas was also distinguished by differences in the populations of neurons that were receptor-bearing. Specifically, whereas all of the ERβ-IR cells in the association cortices were PV-IR, it was only most of the ERβ-IR cells in the sensorimotor areas that contained this calcium binding protein. Thus, in every region evaluated, between 10 and 20% of the ERβ-IR cells in layer V, and roughly half of the ERβ-IR cells found outside of this layer were PV-immunonegative (Fig. 4D–F). As in the association areas, a small number of ERβ-IR cells that were CB-IR were also observed (Fig. 5A,B). While such cells were most common in layer Vb in the association areas, in the sensorimotor fields, they could be found throughout layers II/III and V/VI. Numerically, these ERβ/CB-IR cells did not seem sufficient to account for all ERβ-IR cells left unlabeled by PV in alternate sections.

ERβ in Piriform and Entorhinal Cortex: Regional, Laminar and Cellular
In contrast to the sometimes subtle distinctions that differentiated receptor immunoreactivity among some of the sensory, motor and association regions of the cortex, more noticeable differences in the laminar and cellular distribution of ERβ-IR set off the piriform and entorhinal cortices both from isocortical areas and from one another. For example, while most cortical labeling was concentrated in layer V, in the three-layered piriform cortex ERβ-IR was localized to its deepest layer (layer III, Fig. 7A), while in the entorhinal cortices, ERβ-IR was more evenly scattered across layers III–V (Fig. 7C). Further, while ERβ-IR cells in sensorimotor and association areas were predominantly in and some areas exclusively PV-IR, in the piriform cortex typically less than half of the ERβ-IR cells present within a given field were PV-IR, and in the entorhinal cortices the proportion of ERβ/PV-IR cells dropped from ~50% in the lateral entorhinal area to almost zero in the medial entorhinal cortex (Fig. 7C). Many of the ERβ-IR cells in these regions were,
however, immunoreactive for CB. Thus, in the piriform cortex, as many as two-thirds of the ERβ-IR cells present were also CB-IR (Fig. 7B), and in the entorhinal areas almost all visible receptor-bearing cells were also CB-IR (Fig. 7D).

ERα in the Cerebral Cortex

The distribution, apparent density, and neurochemical signatures of ERα-IR cells in the rat cerebral cortex provided contrasts to corresponding parameters of immunoreactivity for ERβ. As a general rule, for example, ERα-IR cells were noticeably less numerous than ERβ-IR cells. Perhaps as a result of their relatively low densities, it was only in exceptional cases that clear cut regional differences in the distribution or density of ERα-IR cells could be discerned among individual cortical areas. As for ERβ-IR, however, the patterns of ERα-IR in piriform and entorhinal areas were readily distinguished from those in some of the more laterally situated sensorimotor and association areas of the cerebrum. Descriptions of these two areas are thus treated below in separate sections, as they were for ERβ-IR.

ERα in Sensorimotor and Association Areas: Regional, Laminar, and Cellular Distribution

Most sensorimotor and association areas of the cortex had only sparse complements of ERα-IR cells, which were found mainly in layers II/III and V/VI (Figs 8 and 9B). Although few in number, there were hints that ERα cells were not randomly distributed. It was not uncommon, for example, to find a repeated pattern of supragranular ERα labeling that was characterized by small numbers of receptor-positive cells studding the very top of layer II (Figs 8 and 9A,C). The granular and agranular insular cortices stood out, however, as areas where ERα-IR was markedly more dense than in laterally flanking cortical zones, and was more obviously concentrated in layer V (Figs 8 and 9C,D). These features gave ERα-IR in these regions a likeness to ERβ-IR. Double-labeling studies revealed, however, that whereas all of the ERβ-IR cells in these association areas were PV-IR, few to none of the ERα-IR cells identified here – or indeed in any area of the sensorimotor or association areas examined – were PV-IR (Fig. 2O) or CB-IR (Fig. 2M).
Estrogen Receptors in Rat Cerebral Cortex

Kritzer

The studies presented here used immunocytochemistry to map estrogen receptor isoform distribution in the cerebral cortex in mature humans and animals. Behavioral, biochemical, and anatomical studies suggest that estrogen maintains some ability to stimulate structure and/or function in the cerebral cortex in order to gain insight into potential target sites.

Discussion

Behavioral, biochemical, and anatomical studies suggest that estrogen maintains some ability to stimulate structure and/or function in the cerebral cortex in mature humans and animals. The studies presented here used immunocytochemistry to map the distribution of the α and β isoforms of the intracellular estrogen receptor in all major subdivisions of the adult rat cerebral cortex in order to gain insight into potential target sites for relevant receptor-mediated hormone actions. These studies revealed that with only rare exceptions, ERα-IR and/or ERβ-IR cells were present in all major fields of the iso- and allocortex of hormonally intact adult male and female rats. Although both were similarly distributed in males and females, each receptor isoform showed its own unique, highly selective distribution with respect to identified cortical regions, layers, and cells. These distributions were similar to previous immunocytochemical and/or in situ hybridization analyses of receptor subtype-specific proteins and/or mRNAs (Simerly et al., 1990; Shughrue et al., 1997; Weiland et al., 1997; Mufson et al., 1999), thus supporting the specificity of the antibodies used to recognize specific estrogen receptor subtypes. The correspondence with in situ hybridization studies also supports the conclusion that previously localized transcripts may be active in producing receptor proteins. There is less compelling correspondence, however, between the distribution of receptive machinery detected by immunocytochemistry or in situ hybridization and the distribution of cortical cells identified by hormone binding or uptake. Although together the summed patterns of ERα-IR and ERβ-IR observed in this study provide an excellent match for the distribution of cells concentrating the synthetic estrogen [3H]11β-methoxy-17-ethyl estradiol in the cerebral cortex of neonatal rats (Sheridan 1979), receptor immunoreactivity appears to be in excess of maps of [3H]-estradiol or [125I]11β-methoxy-16α-iodoestradiol concentrating cells generated in the cortices of adult rats and mice (Pfaff and Keiner, 1973; Shughrue et al., 1990). Although there may be technical reasons for these spatial mismatches, e.g. the specific activity of radiolabeled probes, until resolved some caution may be warranted in interpreting receptor-enriched loci identified by immunocytochemistry or and/or in situ hybridization alone as sites of functionally patent hormone–receptor interactions.

ERβ-IR and ERα-IR Does not Colocalize with Macroglial Antigens in the Adult Rat Cerebrum

Astrocytes, oligodendrocytes, and microglia have all been identified as reactive to estrogen stimulation in the cerebral or hippocampal cortices (Day et al., 1993; Garcia-Estrada et al., 1993) and/or in culture (García-Segura et al., 1995). The question of whether and which of these cells are actually
estrogen receptor-bearing, however, has only been partially addressed for the cerebral cortex. For example, while previous studies have shown that immunoreactivity for ERα is not colocalized with the astrocyte marker GFAP in the hippocampus or neocortex (Weiland et al., 1997), anatomical relationships between ERα and other glial cell types, e.g. microglia, oligodendrocytes, have only been explored in culture [e.g. Gudino-Cabrera and Nieto-Sampedro and Mor et al.]

**Figure 9.** Representative low power photomicrographs showing the distribution of nuclei immunoreactive for ERα in sensory (A, Par1), motor (B, Fr1) and associational areas (C, AID; D, AIV). Many cortical regions displayed a stereotyped pattern of labeled cells along the very top of layer II that is illustrated in panel (A) [arrowheads, see also panel (C)]. In areas AID (C) and AIV (D), ERα-immunoreactive nuclei were more numerous and uniquely surrounding the rhinal sulcus (see also Fig. 8). The tissue sections shown are from hormonally intact adult male rats. Scale bars: A = 100 µm, B–D = 250 µm.
Previous investigations of ERβ-glial relationships are also limited and to date include only analyses of ERβ-IR and GFAP-IR colocalization in the hippocampus and cerebellum (Azcottia et al., 1999; Jakab et al., 2001). The studies presented here are thus among the first to comprehensively examine the co-distribution of immunoreactivity for ERα, ERβ, oligodendrocytes, and astrocytes in major divisions of the iso- and allocortex. None of these combinations, however, revealed evidence for colocalization between receptor and glial antigens within single cells. It is possible that cortical cells do exist that contain markers for both ER and glia, but which go undetected—perhaps due to low antigen levels. However, this possibility seems remote since the dimensions of cortical ERα-IR and ERβ-IR nuclei were typically larger than the nuclear compartments visible in immunolabeled astrocytes and oligodendrocytes. Although not tested directly, similar arguments suggest that colocalization of ERα and/or ERβ in cortical microglia may be even less likely, as these cells are smaller still than the two macroglial populations examined. Thus, while glial cells in the cerebral cortex may be sensitive to estrogen stimulation, the data presented here and previously suggest that observed effects may be indirect and/or perhaps mediated by receptors other than ERα or ERβ sites.

**ERα-IR and ERβ-IR is Present in Different Subsets of Neurons in the Adult Rat Cerebrum**

The negative findings obtained with respect to glial cell markers stood in sharp contrast to positive findings of colocalization between ERα-IR and immunoreactivity for NSE that surrounded almost every receptor-positive nucleus examined. While previous studies have alluded to the neuronal nature of ER-bearing cortical cells based either on morphology (Pfaif and Keiner, 1973) or by exclusion from studies using glial cell markers (Weiland et al., 1997), the present co-labeling data with NSE-IR provide the first positive demonstrations of the neuronal character of ERα- and ERβ-bearing cells in the rat cerebral cortex. This labeling also revealed morphological clues as to whether receptor-bearing neurons were pyramidal or non-pyramidal. It was immediately evident, for example, that large and medium-sized cortical pyramids—the principal output cells of the cerebral cortex (Peters and Jones, 1984)—were immunonegative for both ERα and ERβ. It was also evident that ERβ-IR was sequestered within non-pyramidal neurons; in virtually every case, ERβ-IR nuclei were found within small NSE-IR somata that radiated either bipolar or multipolar arrays of dendrites that bore striking resemblance to subsets of cortical neurons identified as GABAergic (Meinecke and Peters, 1987).

The characterization of ERα-IR neurons, however, was less clear. While ERα-IR cells residing in upper layers seemed to be non-pyramidal, those in deeper layers tended to display more ambiguous form. Because colocalization studies were limited to antigens preserved in the relatively weak fixative needed to optimize ERβ-IR, double labeling with GABA, glutamic acid decarboxylase (GAD) and glutamate could not be used to clarify whether these cells were small excitatory pyramids and/or inhibitory local circuit neurons. Immunoreactivity for the calcium binding proteins PV and CB, which in rat cortex label subsets of GABAergic interneurons (DeFelipe, 1993), on the other hand, was well preserved. However, double-labeling studies revealed that almost without exception, ERα-IR cortical cells were immunonegative for PV and for CB. In marked contrast, PV-IR identified an overwhelming majority of the ERβ-IR cells in most sensorimotor and association areas, and together with CB seemed to account for most, if not all, of the ERβ-IR cells in the allocortical piriform and entorhinal areas as well.

Localization of the classical estrogen receptor within GABAergic neurons has precedents in other brain structures. In the pre-optic area, for example, the majority of receptor-bearing neurons contain GAD, the synthetic enzyme for GABA (Herbison et al., 1993), while in the hippocampus, the laminar locations of ERα-IR cells identify them as local circuit neurons (Weiland et al., 1997). Thus while neurochemical signatures of ERα-IR neurons in the cerebral cortex were not established in this study, there may be some anticipation that they correspond to subsets of GABAergic, local circuit neurons. Importantly, however, the negative findings obtained with respect to colocalization with CB and PV suggest that whatever their identity, ERα-IR neurons in the cerebral cortex are unlikely to overlap with ERβ-IR cells. Although the possibility for a few individual cases of receptor isoform colocalization cannot be excluded, the present results suggest that at the cellular level, ERα- and ERβ-mediated signaling streams are largely segregated in the adult rat cortex. Accordingly, few if any cortical neurons are likely to utilize estrogen’s added signaling capacity afforded by the heterodimerization of ERα and ERβ subunits (Pettersson et al., 1997; Ogawa et al., 1998). As discussed separately by receptor subtype below, the cellular segregation of ERα versus ERβ signaling pathways appears to be further accentuated by regional and laminar differences in receptor distribution as well.

**Regional and Laminar Localization of ERα-IR in Adult Rat Cortex: Comparison to Previous Studies and Functional Considerations**

The very low levels of the classical ER may have discouraged focus on ERα as a subject for intensive, exclusive investigation in the mature cerebral cortex. Nonetheless, fragmentary data exist describing the distributions of ERα receptor proteins and mRNAs in the adult rat cortex. In most cases these data are consistent with details of the distribution of receptor-like immunoreactivity identified here. For example, the present results are a good match to a previous immunocytochemical study of the rat forebrain which included descriptions of small numbers of immunoreactive cells scattered among layers II/III of the cingulate and piriform cortices, and among layers II/III and IV/V of the lateral neocortex (Mufson et al., 1999). The present findings are also consonant with those from a second immunocytochemical study of rat hippocampus that additionally identified sparse complements of ER-IR cells particularly within the supragranular layers of retrosplenial and parietal cortex (Weiland et al., 1997). Curiously, however, in situ hybridization studies in adult rats consistently indicate that the few ERα-mRNA-containing cells identified are present mainly in layers IV (Simerly et al., 1990; Shughrue et al., 1997)—a layer in which neither ERα-IR nor ERβ-IR cells were observed in this study.

While many studies have emphasized the paucity and scatter of cortical ERα-IR cells, some novel features of ERα-IR identified here suggest precision in the distribution of this receptor isoform in the adult cerebrum. First, while perhaps scattered tangentially, in most areas ERα-IR cells took up highly stereotyped sublaminal locations, including the formation of a tier of receptor-positive cells that stood out along the superficial margins of layer II. Further, the insular cortices were unique sites of abrupt increases in the apparent density of ERα-IR cells, and dramatic shifts in their laminar distribution. Thus, while perhaps unimpressive for their number, the laminar locations and regional concentrations of ERα-bearing neurons suggests that they may nonetheless hold well-defined positions with respect to...
functionally specialized cortical subdivisions and circuits. The precise positioning of receptor-bearing neurons in the supragranular layers may further suggest some specific connection to the corticocortical communications that are mediated in part within these layers. Establishing the identity of ERα-IR cells, e.g. as projection or local circuit neurons, will be important next steps in drawing further inference about the cortical circuits and processes that may be direct targets of estrogen stimulation at the classical ER site.

**Regional and Laminar Localization of ERβ-IR in Adult Rat Cortex: Comparison with Previous Studies and Functional Considerations**

The mRNAs encoding the ERβ-receptor subtype, and, more recently, the receptor proteins themselves have been widely mapped in regions of the adult rat brain including the cerebral cortex (Shughrue et al., 1997; Shughrue and Merchenthaler, 2001). When compared with the present data, both descriptions reveal points of common ground as well as some disparities between the two types of analysis. Regionally, for example, immunocytochemistry and in situ hybridization are generally well aligned. Thus, in the isocortex, both ERβ-mRNA and ERβ-IR cells are localized mainly in deep cortical layers, and tend to be more abundant in lateral (sensorimotor) compared with medial (association) areas. Similarly, in the entorhinal cortex, receptor immunoreactivity and mRNAs are dense but more broadly distributed over major cellular layers (Shughrue et al., 1997). As for ERα, however, there are some discrepancies regarding the precise laminar distribution of immunoreactive and in situ hybridization signals for ERβ. In all but the piriform and entorhinal cortices, for example, cells expressing ERβ mRNAs are described as being present in layers IV–VI, while the ERβ-IR cells identified here seemed to be more concentrated in (and in some cases, confined to) layer V, were virtually never seen in layer IV and were only rarely encountered in VI.

The recent findings of a relative abundance of ERβ in the cerebral cortex has brought attention to this receptor subtype in particular as potentially explaining some of estrogen’s effects in the adult cerebrum (see Kuiper et al. (Kuiper et al., 1998)). The precise laminar and cellular localization of ERβ-IR identified in this study add details that may be useful in further developing such functional hypotheses. For example, in the isocortex, ERβ-IR neurons were concentrated in layer V, an infragranular stratum housing cortical outputs to subcortical motor centers such as the spinal cord, tectum, and neostriatum. Double-labeling studies indicated, however, that it is not the projection neurons themselves that are direct targets for receptor-mediated estrogen stimulation, but rather, subsets of the inhibitory interneurons that modulate their activity. Specifically, ERβ-IR was present within interneurons that were largely immunoreactive for PV. This particular neurochemical signature in turn reveals the location of hormone signaling machinery as local circuit neurons that respond to depolarization with fast spiking, slowly adapting activity (Kawaguchi and Kubota, 1993) and which are presumably basket and chandelier cells (see DeFelipe (DeFelipe 1993)); the latter are identifications that bring with them additional knowledge regarding stereotyped synaptic relationships and postulated roles in divisive and subtractive forms of inhibition (Blomfield, 1974; Somogyi et al., 1982). This information may combine with the regional differences in laminar distribution and areal densities of the PV cells observed in this study as receptor-bearing to produce specific predictions about the computations and functions that are directly influenced by estrogen activation at ERβ sites in the adult isocortex.

Although the specific technical support of Mr. Alex Adler is gratefully acknowledged, Mr. James Tsakos is also thanked for his assistance in initiating these studies. This work was supported by FIRST Award (R29NS35422) to MFP.

**Notes**

The outstanding technical support of Mr. Alex Adler is gratefully acknowledged. Mr. James Tsakos is also thanked for his assistance in initiating these studies. This work was supported by FIRST Award (R29NS35422) to MFP.

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