Sex in the brain. Gender differences in the human hypothalamus and adjacent areas. Relationship to transsexualism, sexual orientation, sex hormone receptors and endocrine status
Kruijver, F.P.M.

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CHAPTER

ESTROGEN-RECEPTOR-β DISTRIBUTION IN THE HUMAN HYPOTHALAMUS: SIMILARITIES AND DIFFERENCES WITH ERα DISTRIBUTION

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²Department of Psychiatry, Leiden University Medical Centre (LUMC), 2300RC Leiden, The Netherlands
³Rijnsgeest Group (RGG), 2300RC Leiden, The Netherlands
ABSTRACT

This study reports the first systematic rostrocaudal distribution of estrogen receptor beta immunoreactivity (ERβ-ir) in the human hypothalamus and adjacent areas in five males and five females between 20–39 years of age and compares its distribution to previously reported ERα in the same patients. ERβ-ir was generally observed more frequently in the cytoplasm than in the nucleus and appeared to be stronger in women. Basket-like fiber stainings, suggestive for ERβ-ir in synaptic terminals, were additionally observed in various areas. Men showed more robust nuclear ERβ-ir than women in the medial part of the bed nucleus of the stria terminalis, paraventricular and paratenial nucleus of the thalamus, while less intense, but more nuclear, ERβ-ir appeared to be present in, e.g., the BSTc, sexually dimorphic nucleus of the medial preoptic area, diagonal band of Broca and ventromedial nucleus. Women revealed more nuclear ERβ-ir than men of a low to intermediate level, e.g., in the suprachiasmatic, supraoptic, paraventricular, infundibular, and medial mamillary nucleus. These data indicate potential sex differences in ERβ expression. ERβ-ir expression patterns in subjects with abnormal hormone levels suggests that there may be sex differences in ERβ-ir that are “activational” rather than “organizational” in nature. Similarities, differences, potential functional, and clinical implications of the observed ERα and ERβ distributions are discussed in relation to reproduction, autonomic-function, mood, cognition, and neuroprotection in health and disease. J. Comp. Neurol. 466:251–277, 2003.

In 1996 a novel estrogen receptor which binds 17-β estradiol with a similar affinity as the “classical” estrogen receptor was discovered and assigned estrogen receptor beta (ERβ) (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). ERβ is transcribed from a distinct gene on chromosome 14 at 14q22-24, while human ERβ has been located on the long arm of chromosome 6 (Enmark et al., 1997). The two estrogen receptor (ER) subtypes have ~16% homology in the N-terminal region, 95%homology in the DNA-binding domain, 55% homology in the ligand-binding domain, while only 18% homology is present in the C-terminal domain (Kuiper et al., 1996). Subsequently, various isoforms of ERβ (i.e., ERβ1) have been identified, of which ERβ2 has a lower affinity for estrogens (Chu and Fuller, 1997) and an 18-amino acid insertion in the ligand-binding domain (Maruyama et al., 1998; for overview, see Saunders, 1998; Price et al., 2000; Österlund and Hurd, 2001). Since the identification of ERβ, various studies have investigated its different isoforms, functions, and differences in distribution pattern from the “classical” estrogen receptor alpha (ERβ) in tissue of both the central nervous system and peripheral reproductive as well as nonreproductive organs (see, e.g., Lubahn et al.,
Estrogen receptors (ERs) belong to a receptor superfamily of ligand-inducible transcription factors that comprises androgen-, progesterone-, glucocorticoid hormone-, thyroid hormone-, retinoic acid-, vitamin D-, and orphan receptors (Kawata, 1995). Human and rodent ERα and ERβ can form homodimers as well as heterodimers within target cells, which can bind to specific DNA estrogen response elements (EREs) of target genes. Moreover, both human and rodent ERβ are able to inhibit ERα transcriptional activity and to decrease its overall cellular sensitivity to estradiol at subsaturating hormone levels (Paech et al., 1997; Hall and McDonnell, 1999). A crucial function of ERβ thus appears to modulate ERα transcriptional activity. The relative expression level of ERα and ERβ will, therefore, be a key determinant of cellular responses to agonists or antagonists (cf. Paech et al., 1997; Hall and McDonnell, 1999).

The hypothalamus and other limbic areas play a crucial role in sexual behavior (Segovia and Guillamo’n, 1997; Kawata, 1995). The hypothalamus is not only the center for the neuroendocrine regulation of reproduction, but
also for autonomic functions, biological rhythms, eating, drinking, mood, and cognition (Segovia and Guillamo'n, 1997; Kawata, 1995; Swaab, 1997; McEwen and Alves, 1999; Saper, 2000; Savic et al., 2001; Quadros et al., 2002; Simerly, 2002). The interaction between sex hormones and their receptors may play an important role in these functions and so in the prevalence of related neurological, neuroendocrine, and psychiatric diseases (Swaab and Hofman, 1995; McEwen, 1999; Mufson et al., 1999; Liu et al., 2000; Schumacher et al., 2000; Österlund and Hurd, 2001; Zhou et al., 2001; Kruijver and Swaab, 2002; Swaab et al., 2000, 2001, 2002; Kruijver et al., 2000, 2001, 2002; Gooren and Kruijver, 2002).

Since the early 1960s, binding studies, in situ hybridization, and immunocytochemical studies have revealed a broad range of estrogen responsive target areas in many organs of different vertebrates (reviewed by McEwen and Alves, 1999). These sites not only include reproductive tracts and brain areas involved in reproduction, but also nonreproductive organs and brain areas (Pfaff and Keiner, 1973; Pfaff et al., 1976; McEwen, 1999; Beyer, 1999; Kawata, 1995; Garcia-Segura et al., 2001; Kruijver et al., 2002). It has become clear from many experimental studies during the last decades that especially estrogens but also androgens play a crucial role in sexual differentiation of the developing nervous system by their “organizing” effects and in adult brain function by their “activating” effects (see, e.g., Koch and Ehret, 1989; Breedlove, 1992; Kawata, 1995; Panzica et al., 1995; Cooke et al., 1998; Gibbs and Aggarwal, 1998; McEwen, 1999; Beyer, 1999; Behl and Holsboer, 1999; Ishunin et al., 2000; Balthazar et al., 2001; Gahr, 2001; Kruijver et al., 2001; McCarthy et al., 2002). In order to reveal sensitivity of the adult brain to androgens, proges-

**TABLE 1. Clinicopathological Data of Reference Subjects**

<table>
<thead>
<tr>
<th># NBB</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PMD (hours/min)</th>
<th>FT (days)</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>86032</td>
<td>F</td>
<td>33</td>
<td>&lt;41:00</td>
<td>20</td>
<td>Adenocarcinoma of the lung with several metastases</td>
</tr>
<tr>
<td>80008</td>
<td>F</td>
<td>35</td>
<td>8:00</td>
<td>26</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>85027</td>
<td>F</td>
<td>29</td>
<td>13:10</td>
<td>60</td>
<td>Hepatic coma by alcohol abuse</td>
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<tr>
<td>97055</td>
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<td>39</td>
<td>72:00</td>
<td>35</td>
<td>Aneurysm of the right middle cerebral artery bleeding in ventricle system, herniation of the medulla oblongata</td>
</tr>
<tr>
<td>85041</td>
<td>F</td>
<td>28</td>
<td>5:25</td>
<td>44</td>
<td>Cardiogenic shock by acute myocardial infarction</td>
</tr>
<tr>
<td>94040</td>
<td>M</td>
<td>20</td>
<td>8:00</td>
<td>82</td>
<td>B-cell non-Hodgkin lymphoma, viral pneumonia</td>
</tr>
<tr>
<td>82020</td>
<td>M</td>
<td>27</td>
<td>&lt;41:00</td>
<td>40</td>
<td>Sepsis, pneumonia pericarditis, cerebral edema</td>
</tr>
<tr>
<td>97075</td>
<td>M</td>
<td>33</td>
<td>18:45</td>
<td>32</td>
<td>Multiple cranium fracture, subdural hematoma cranial edema,</td>
</tr>
<tr>
<td>84023</td>
<td>M</td>
<td>37</td>
<td>31:25</td>
<td>100</td>
<td>Bronchopneumonia, pleurisy, lung abscess</td>
</tr>
<tr>
<td>88035</td>
<td>M</td>
<td>23</td>
<td>&lt;65:00</td>
<td>29</td>
<td>Acute myocardial infarction, familial hypercholesterolemia</td>
</tr>
<tr>
<td>80002 (S8)</td>
<td>F</td>
<td>46</td>
<td>2:30</td>
<td>36</td>
<td>Bilateral ovariectomy, ovarian carcinoma</td>
</tr>
</tbody>
</table>

Clinicopathological Data of Subjects with Different Estrogen Levels

<table>
<thead>
<tr>
<th># NBB</th>
<th>Sex</th>
<th>Age (years)</th>
<th>PMD (hours/min)</th>
<th>FT (days)</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>84020 (T1)</td>
<td>MFT</td>
<td>50</td>
<td>ND</td>
<td>30</td>
<td>Suicide</td>
</tr>
</tbody>
</table>

terone, and estrogens in health and disease we set up a series of investigations to study in the human hypothalamus and adjacent brain areas the distribution of androgen, progesterone, and estrogen receptors at the protein level by using immunocytochemistry (ICC) (Fernández-Guasti et al., 2000; Ishunina et al., 2000, 2002; Kruijver et al., 2001, 2002; Ishunina and Swaab, 2001; Kruijver and Swaab, 2002). The human hypothalamic distribution of the androgen and "classical" estrogen receptor alpha (ERα) in relation to sex and endocrine status have been described in separate articles (Fernández-Guasti et al., 2000; Ishunina et al., 2000; Kruijver et al., 2001, 2002; Kruijver and Swaab, 2002).

The present immunocytochemical study was aimed to systematically examine the rostrocaudal distribution of estrogen receptor beta (ERβ) in the human hypothalamus and its relation to sex and endocrine status. Special attention is paid to the similarities and differences with ERα distribution. Potential functional and clinical implications are discussed in relation to reproduction, autonomic function, mood, cognition, and neuroprotection.
MATERIALS AND METHODS

Subjects

The brains of 10 control subjects (five men and five women), ranging from 20–39 years of age, were obtained at autopsy within the framework of The Netherlands Brain Bank. Permission was obtained for a brain autopsy and for the use of tissue and medical records for research purposes. In addition, three age-matched subjects (T1, S2, S8) with abnormal circulating levels of estrogens were investigated (Table 1). For this purpose we studied a 50-year-old castrated and estrogen-treated male-to-female transsexual (T1; #84020), a 31-year-old man (S2; #91005) with a feminizing adrenalcortical carcinoma (which produced very high estrogen levels) and a 46-year-old woman (S8; #80002) with bilateral ovariectomy. T1 was castrated 6 years before death and the last 2 years before death this subject was treated only with estrogens (Ethinyloestradiol 50 μg 2 dd). For more details regarding subjects T1 and S2, see Kruijver et al. (2000); for clinicopathological data, see Table 1. General pathology and neuropathology were performed either at the Free University of Amsterdam (Dr. W. Kamphorst, Prof. F.C. Stam, or Prof. P. van der Valk) or at the Academic Medical Center of the University of Amsterdam (Prof. D. Troost). The control subjects had no primary endocrine, neurological, or psychiatric disease. The stage of the menstrual cycle of the women was not known.

Histology and immunohistochemistry

After autopsy the hypothalamus was fixed in 4% formaldehyde at room temperature (for fixation time, see Table 1), dehydrated, and embedded in paraffin. Serial 6-μm frontal sections were cut on a Leitz microtome.

Paraffin-embedded sections of human hypothalamus were mounted on SuperFrost/Plus (Menzel, Germany) slides and dried overnight on a hot plate at 58°C followed by 24–36 hours in an oven at 37°C. Along the hypothalamic rostrocaudal axis each 100th section was selected and mounted for estrogen receptor beta staining (ERβ-ir). The sections were: 1) deparaffinized and dehydrated by a series of decreasing ethanol concentrations; 2) rinsed in distilled water (2 x 5 min); 3) rinsed in Tris-buffered saline (TBS)-high salt (0.05M; Tris 3.8% NACL; pH 7.6; 2 x 5 minutes; 4) microwave pretreatment in 0.1 M citric acid monohydrate solution (pH 6.0; see Kruijver et al., 2001) 2 x 5 minutes at 700 watts; 5) washed in TBS-high salt for 10 minutes; 6) incubated in TBS-milk for 1 hour at room temperature (RT) (TBS-milk TBS buffer: 0.05M, Tris 0.9% NACL containing a 5% TBS-milk powder dilution (w/v), pH 7.6 (commercial milk powder by ELK, Campina Melkunie, Eindhoven, The Netherlands)); 7) washed in TBS-high salt 1 x 5 minutes; 8) incubated with a primary polyclonal goat anti-ERβ antibody that is directed to the amino-terminus of human ERβ (catalog no. sc-6820 [N-19], Santa Cruz Biotechnology, Santa Cruz, CA; additional controls for specificity of this antibody were performed in the framework of our studies [see below]), diluted 1:50 in Supermix (0.25
TABLE 2: Distribution and Sex Differences in Nuclear, Cytoplasmic, and Fiber ERβ-ir of the Human Hypothalamus

<table>
<thead>
<tr>
<th>(Peri)-hypothalamic area</th>
<th>Men</th>
<th>Women</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>c</td>
</tr>
<tr>
<td>Preoptic region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DBB (CH2)</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>NBM (CH4)</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>hDBB (CH3)</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>Cal</td>
<td>(+4/5)</td>
<td>(-4/5)</td>
</tr>
<tr>
<td>LS</td>
<td>(+5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>MS n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>BSTm</td>
<td>(+5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>BSTc</td>
<td>(+5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>BSTI</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>CDM</td>
<td>(-5/5)</td>
<td>(-5/5)</td>
</tr>
<tr>
<td>ic</td>
<td>(-4/5)</td>
<td>(+4/5)</td>
</tr>
<tr>
<td>ZI</td>
<td>(+4/5)</td>
<td>(+4/5)</td>
</tr>
<tr>
<td>EGP</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>SDN-mPOA</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>mPOA</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>DPc</td>
<td>(5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>VPe</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>SCN</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>PVN</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>SON</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>Tuberal region</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PT</td>
<td>(+5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>PV</td>
<td>(+5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>BSTP</td>
<td>(+5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>DMN</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>VMN</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>INF</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>NTL</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>TM</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>LHA</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>Mamillary region</td>
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<td></td>
</tr>
<tr>
<td>LNM</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
<tr>
<td>MMN</td>
<td>(-5/5)</td>
<td>(+5/5)</td>
</tr>
</tbody>
</table>

Median intensity of label for ERβ-ir in various hypothalamic and adjacent brain structures. The category assigned to a given brain region corresponds to the predominant cell type according to the following scale: - no staining, + = diffuse staining, ++ = nontransparent but individual granules of the reaction product still distinguishable, and +++ = intense opake staining. n = nuclear staining, c = cytoplasmic staining. Fiber staining according to the following scale: - = no positive fibers present, + = few fibers present, ++ = moderate amounts of fibers, and +++ = many fibers present. N.d: not determined. Proportions in parentheses indicate number of patients stained/total number. The hypothalamus is subdivided into its three main regions, which are the preoptic, tuberal, and mamillary region, according to most authors (Saper, 1990).

g gelatin and 0.5 ml Triton X-100 in 100 ml TBS, pH 7.6) for 1 hour at room temperature (RT) and left overnight at 4°C. The next day the sections were: 9) washed in TBS-milk 3 x 10 minutes; 10) washed in TBS-high salt 2 x 5 minutes; 11) incubated with secondary biotinylated antirabbit IgG (Vector Laboratories, Burlingame, CA) 1:200 in Supermix for 1 hour at RT; 12) washed in TBS-milk 3 x 10 minutes; 13) washed in TBS-high salt for 5 minutes; 14) incubated with avidinbiotin complex (ABC) (Elite kit, Vector Laboratories, Burlingame, CA) 1:800 in Supermix for 1 hour at RT; 15) rinsed in TBS-high salt (pH 7.6) 3 x 5 minutes; 16) incubated with biotinylated tyramine diluted 1:500 in TBS plus 0.01% hydrogen peroxide (H2O2) for 15 minutes at room temperature; 17)
<table>
<thead>
<tr>
<th>Hypothalamic Area</th>
<th>Men ERβ</th>
<th>Men ERα</th>
<th>Women ERβ</th>
<th>Women ERα</th>
</tr>
</thead>
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<tr>
<td>Preoptic region</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>DBB (CH2)</td>
<td>+(5/5)</td>
<td>-(5/5)</td>
<td>+(5/5)</td>
<td>+(4/5)</td>
</tr>
<tr>
<td>NBm (CH4)</td>
<td>-(5/5)</td>
<td>+(5/5)</td>
<td>-(5/5)</td>
<td>-(5/5)</td>
</tr>
<tr>
<td>hDBB (CH3)</td>
<td>-(5/5)</td>
<td>++(5/5)</td>
<td>-(5/5)</td>
<td>++(5/5)</td>
</tr>
<tr>
<td>Ca</td>
<td>+(4/5)</td>
<td>++(4/5)</td>
<td>-(4/5)</td>
<td>+(4/5)</td>
</tr>
<tr>
<td>LS</td>
<td>+(5/5)</td>
<td>+(5/5)</td>
<td>+(4/5)</td>
<td>++(4/5)</td>
</tr>
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<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>BSTm</td>
<td>+(5/5)</td>
<td>++(5/5)</td>
<td>-(5/5)</td>
<td>+(5/5)</td>
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<tr>
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<td>+(5/5)</td>
<td>+(5/5)</td>
<td>-(5/5)</td>
<td>+(5/5)</td>
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<td>BSTI</td>
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<td>-(5/5)</td>
<td>-(5/5)</td>
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</tr>
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<td>-(5/5)</td>
<td>+(5/5)</td>
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<td>+(5/5)</td>
<td>+(5/5)</td>
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<td>+(5/5)</td>
<td>+(5/5)</td>
<td>+(5/5)</td>
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<td>Tuberal region</td>
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</tr>
<tr>
<td>PT</td>
<td>++(5/5)</td>
<td>+(5/5)</td>
<td>-(4/5)</td>
<td>+(4/5)</td>
</tr>
<tr>
<td>PY</td>
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<td>+(5/5)</td>
<td>-(4/5)</td>
<td>+(4/5)</td>
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<td>-(4/5)</td>
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<td>-(5/5)</td>
<td>-(5/5)</td>
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<tr>
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<td>+(5/5)</td>
<td>-(5/5)</td>
<td>-(5/5)</td>
</tr>
<tr>
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<td>-(5/5)</td>
<td>-(5/5)</td>
<td>-(5/5)</td>
<td>-(5/5)</td>
</tr>
<tr>
<td>TM</td>
<td>-(5/5)</td>
<td>+(5/5)</td>
<td>-(4/5)</td>
<td>+(4/5)</td>
</tr>
<tr>
<td>LHA</td>
<td>-(5/5)</td>
<td>+(5/5)</td>
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Median intensity of label for ERβ-ir in various hypothalamic and adjacent brain structures. The category assigned to a given brain region corresponds to the predominant cell type according to the following scale: - = no staining, + = staining diffuse and transparent, ++ = staining not transparent but individual granules of the reaction product still distinguishable, and +++ = intense opaque staining. n = nuclear staining, c = cytoplasmic staining. Fiber staining according to the following scale: - = no positive fibers present, + = few fibers present, ++ = moderate amounts of fibers, and +++ = many fibers present. N.d.: not determined. Proportions in parentheses indicate number of patients stained/total number. The hypothalamus is subdivided into its three main regions, which are the preoptic, tuberal, and mammillary region, according to most authors (Saper, 1990).

Washed in TBS-high salt 3 x 5 minutes; 18) incubated with ABC complex as described above; 19) rinsed in 0.05 mol/L Tris-HCL (pH 7.6); 20) incubated in Tris-HCL (pH 7.6) containing 0.5 mg/ml 3,3-diaminobenzidine (Sigma Chemicals, St. Louis, MO), 0.01% H2O2 and 0.2% nickel ammonium sulfate; 21) washed in Tris-HCL 2-10 minutes; 22) dehydrated in graded ethanol, cleared in xylene, and coverslipped with Etellan mounting medium (Merck, Darmstadt, Germany).
**TABLE 4. Distribution and Sex Differences in Cytoplasmic ERβ-ir versus Cytoplasmic ERα-ir**

<table>
<thead>
<tr>
<th>(Peri)-hypothalamic area</th>
<th>Men ERβ</th>
<th>Men ERα</th>
<th>Women ERβ</th>
<th>Women ERα</th>
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<tr>
<td>hDBB (CH3)</td>
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<td>+(5/5)</td>
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</table>

Median intensity of cytoplasmic label for ERβ-ir and ERα-ir in various hypothalamic and adjacent brain structures. The category assigned to a given brain region corresponds to the predominant cell type according to the following scale: = no staining, + = staining diffuse and transparent, ++ = staining nontransparent but individual granules of the reaction product still distinguishable, and +++ = intense opaque staining. n = nuclear staining. N.d: not determined. Proportions in parentheses indicate number of patients stained/total number. The areas in bold point to reversed sex differences for ERβ-ir and ERα-ir. The hypothalamus is subdivided into its three main regions.

**Specificity**

A number of antibody specificity tests on the ERα and ERβ antibodies used (Santa Cruz Biotechnology, cat. #sc-542 (ERα) and #sc-6820 (ERβ)) have been reported in the literature (Fitzpatrick et al., 1999; O'Brien et al., 1999; Azcoitia et al., 1999; Clarke et al., 2000; Saji et al., 2000; Ishunina et al., 2000; Su et al., 2001; Kruijver and Swaab, 2002; Kruijver et al., 2002). No cross-reactivity of the ERα antibody MC-20 with ERβ proteins of rat ovary granulosa cells and uterus tissue were observed (see O'Brien et al., 1999, and Clarke et al., 2000,
ERβ Nuclear

**Males**

**Females**

Fig. 3. Frontal sections from rostral to caudal through the human hypothalamus. These schemes illustrate the distribution of the median values of the intensity of nuclear ERβ staining in young men (n = 5) and women (n = 5) between 20-40 years of age. Note the presence of region-dependent sex differences.

respectively). For instance, ERα specificity of MC-20 and the absence of cross reactivity with the ERβ was provided by Clarke et al. (2000), who showed a 68 kD prominent specific band for ERα in a Western blot of pregnant rat uterine tissue. We found a similar 68 kD prominent specific band for ERα in Western blots of human hypothalamic tissue with the same antibody (Kruijver et al., 2002; Fig. 2). The antibody MC-20 specifically recognized baculovirus expressed human ERα, but not ERβ protein in an electrophoretic mobility shift assay (Fitzpatrick et al., 1999). A number of observations on the specificity of the ERβ-antibody (N-19) used by us have been added to the literature.
ERβ Cytoplasmic/Fibre

Males

Fig. 4. Frontal sections from rostral to caudal axis through the human hypothalamus. These schemes illustrate the distribution of the median values the intensity of cytoplasmic and fiber (open dots) ERβ staining in young men (n = 5) and women (n = 5) between 20–40 years of age. Note the presence of region-dependent sex differences.

In a spot blot test, as previously described by Fernández-Guasti et al. (2000), according to the technique of van der Sluis et al. (1988), N-19 was shown to recognize its blocking peptide on nitrocellulose paper revealing the expected concentration gradient (Santa Cruz Biotechnology, blocking peptide cat. #sc-6820 P). Subsequently, preadsorption of the N-19 antibody with this peptide was carried out and the staining protocol was performed on hypothalamic sections. After omission of the first antibody as well as after preadsorption of the ERβ antibodies with the peptide to which it was raised, all nuclear, cytoplasmic, and neuritic immunoreactivities were abolished (Fig. 1). A Western blot with the
ERα antibody MC-20 on human hypothalamic tissue showed a specific band around the expected 68 KDa of ERα with no such band around the 54 kD of ERβ (Fig. 2; see Kuiper et al., 1996; Clarke et al., 2000; Kruijver et al., 2002). Furthermore, two different ERβ antibodies, N-19 (N-terminus directed) and L-22 (C-terminus directed), displayed similar distribution patterns on sections of the human hypothalamus (see also Ishunina et al., 2000). The differences in distribution revealed by the ERα antibody MC-20 and the ERβ antibody N-19 in the hypothalamus (Figs. 5, 6; Tables 3, 4), pituitary, ovary, and testis also support the specificity of these antibodies (see Ishunina et al., 2000; Kruijver and Swaab, 2002; Kruijver et al., 2002).

The differences between the staining patterns of the two ERs in the pituitary, ovary, and testis have been reported previously (Ishunina et al., 2000) and were in good agreement with those reported in the literature (e.g., Mitchner et al., 1998; Fitzpatrick et al., 1999; Pelletier and El-Alfy, 2000). For instance, the pituitary showed moderate nuclear and strong cytoplasmic ERα staining, whereas only weak cytoplasmic ERβ staining was observed (confirming similar data by Mitchner et al., 1998; Kruijver et al., unpubl. obs.; data not shown). Moreover, in the study of Ishunina et al. (2000), in which the same ERα (MC-20) and ERβ (N-19) antibodies were used on the supraoptic nucleus (SON), an opposite change in staining was found according to sex and age for ERα and ERβ. Young men differed from age-matched women in having stronger nuclear ERα staining with much less nuclear ERβ staining. A shift from neuronal nuclear to cytoplasmic ERβ was observed in the SON of postmenopausal women, whereas the reversed pattern from cytoplasmic to nuclear staining for ERα-ir occurred following menopause. Together, these data demonstrate the specificity of the two antibodies used.

A Western blot was performed on human brain hypothalamic tissue in order to determine whether the ERβ-antibody (N-19) would recognize a protein band around the expected 54 kD weight (Kuiper et al., 1996).

**Western blotting procedure.**

Hypothalamic tissue of a control patient (NBB patient #96-052) was obtained at autopsy according to the Netherlands Brain Bank protocol, quickly frozen in liquid nitrogen, and subsequently stored at −80°C.

A sample of ~1 mm3 of hypothalamic tissue was taken and transferred to an Eppendorf tube containing 500 μl suspension buffer (0.1M NaCl, 0.01M Tris-HCl (pH 7.6), 0.001M EDTA (pH 8.0), phenylmethylsulfonyl fluoride (100 μg/ml), leupeptin (10 μg/ml)). The tissue was homogenized using an ultra-turrax and spun down for 5 minutes at 14,000 rpm, 4°C. Protein concentration of the sample was measured in a Bradford assay using bovine serum albumin standards. The sample was diluted in suspension buffer to a final concentration of 133 μg/ml. The protein sample was then mixed with an equal volume of 2 loading buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.2% bromophenol blue,
20% glycerol; resulting in a final concentration of 1 g protein per lane), boiled for 5 minutes, loaded on a 7.5% SDS polyacrylamide gel, and run at 30 mA, 250W in running buffer (Tris-HCl 0.75M, pH 8.8). Gels were semi-dry blotted (Whatmann filter paper was soaked in Townin buffer (0.025M Tris-HCl, 0.2M glycine, methanol 20% v/v) on nitrocellulose paper (pore size 0.45 m) for 1 hour at 100 mA, 100V, 250W, in sumi-milk (0.25% [w/v] gelatin, 0.5% [v/v] Triton X-100, 0.5% [w/v] milkpowder in TBS, pH 7.6) for 1 hour at RT, incubated in anti-ERβ antibody 1:800 (N-19 Santa Cruz) in sumi-milk for 1 hour at RT (room temperature) followed by an overnight incubation at 4°C). The next day the nitrocellulose sheets were rinsed in TBS-Tween (TBST), 2 x 10 minutes, incubated in the second conjugated antibody, swine antirabbit immunoglobulins conjugated to HRP (horse radish peroxidase) (DAKO, Carpinteria, CA, P0449) 1:1,000 in TBST for 1 hour at RT, rinsed in TBST 2 x 10 minutes, and incubated in Lumi Light Western Blotting Substrate solution (Roche, Almere, The Netherlands) for 5 minutes under dark conditions. For detection of the signal the nitrocellulose sheets were exposed to Lumi Chemiluminescent Detection Film (Boehringer Mannheim, Germany) for 25 minutes, developed in D-19 developer (Kodak) for 4 minutes, rinsed in running tap water, and fixed in Maxfix (Kodak).

As a negative control, blots from the same run, containing the same hypothalamus sample, were incubated in sumi-milk, without primary antibody. The next day they were rinsed in TBST and processed together with the other blot. Incubating the blots with the ERβ-antibody sumi-dilution resulted in a band at 54 kD, whereas omission of the antibody resulted in a negative blot (see Fig. 2). No such band was seen around the 68 kDa level of ERα, as was previously shown by Kruijver et al. (2002), which further supports the specificity of N-19 for ERβ (see Fig. 2).

Analysis of the ERβ staining intensity

The sections were rated for staining intensity by three independent investigators, who were in the verification process blind to the subjects sex or endocrine status, according to the same methods as previously described by Fernández-Guasti et al. (2000) and Kruijver et al. (2002). The few differences in rating (less than 5% of all areas studied and concerning at most one (+) difference in rating scale) were agreed by settlement. The category assigned to a given brain region corresponded to the predominant cell type within the field of view according to the following scale: − = no staining, + = staining diffuse and transparent, ++ = staining non transparent but individual granules of the reaction product still distinguishable, and +++ = intense opaque staining. The staining range was established for both the cytoplasm and the nucleus and the median scores (conform the procedures described in Fernández-Guasti et al., 2000; Kruijver et al., 2002) are given in Table 2, 3, and 4. Fiber staining was rated according to the following scale: − = no positive fibers present, + =
Distinct expression patterns in the human hypothalamus and adjacent areas of ERα-ir and ERβ-ir as illustrated for the BSTc (A, B), SON (C, D), TM (E, F), and LMN (G, H). ERα (A) versus ERβ staining in BSTc (B). Note the pericellular basket-like ERα-ir suggestive of ERβ-positive terminals in B. C, D: Robust cytoplasmic and nuclear ER (C) versus low nuclear and cytoplasmic ERβ staining in SON neurons (D). E, F: Nuclear and cytoplasmic ERα-ir (E) in contrast to the lack of nuclear and weak cytoplasmic ER staining in TM neurons (F). ERα (G) versus ER staining in LMN neurons (H). Note the strong nuclear and cytoplasmic ERα staining in LMN neurons (G) and the clear lack of such strong cellular staining for ERβ (H). Also note the stronger perivascular ERβ staining in H versus G and the stronger cytoplasmic ERβ-ir in the female TM neurons (H) versus male TM neurons (F). Scale bar =100 μM in H (applies to C–H); 25 μm for A, B.
few fibers present, ++ = moderate amounts of fibers, and +++ = many fibers present. The estimates were made at three different microscopic magnifications using x2.5, x10, and x40 objectives. The brain regions containing ER-ir were identified with the help of maps of coronal sections of the human and primate brain (Mesulam et al., 1983; Mai et al., 1997) and by antipeptide antibodies as markers for the different hypothalamic cell groups (Swaab, 1997).

Statistics

Group comparisons for postmortem delay (PMD), fixation time (FT), or age were made with the nonparametric Mann-Whitney U-test at the two-tailed level. Differences were considered significant at P < 5%.

Photomicrographs

Photomicrographs were made with the help of an IBAS image-analysis system (Kontron Elektronic, Munich, Germany).

RESULTS

Specificity of the ERβ-staining

No statistical differences were present between males and females in postmortem delay (PMD), fixation time (FT) or age (P = 0.70, P = 0.35, and P = 0.20, respectively), so that the reported sex differences cannot be confounded by these parameters. Neither a long PMD (up to 72 hours) nor a long FT (up to 100 days) appeared to affect the ER-ir. For instance, patient #80008 had a lower nuclear ERβ expression pattern than patient #85041, who displayed a clearly stronger ERβ expression pattern, despite the fact that their postmortem times were rather similar, with an even longer fixation time of patient #85041. No relationship was observed between the cause of death and ERβ-ir. However, ERβ-ir appeared to be dependent on the presence or absence of circulating levels of estrogens (see below).

ERβ distribution in the human hypothalamus

The results of scoring the intensity of nuclear and cytoplasmic ERβ-immunoreactivity (ir) and fiber ERβ-ir is extensively given in Table 2 and Figures 3 and 4. In summary, ERβ-ir was observed more frequently in the cytoplasm than in the nucleus. Predominant cytoplasmic ERβ-ir was observed in cells of the choroid plexus and in neurons of the lateral bed nucleus of the stria terminalis (BST1), the medial preoptic area (mPOA), the nucleus basalis of Meynert (NBM or Ch4), the horizontal diagonal band of Broca (hDBB or Ch3), the dorsomedial nucleus, the tuberomammillary nucleus (TM), the lateral hypothalamic area (LHA), and the lateromammillary nucleus (LMN) (see Table 2, Fig. 4).

Low to intermediate nuclear ERβ-ir was observed in men and women in neurons of various brain areas (see Table 2, Fig. 4). The nucleus accumbens
(Ac) displayed nuclear ER\(\beta\)-ir in the few patients in which this structure was available (see Fig. 13A). ER\(\beta\)-ir was not only observed in neurons, but also in endothelial cells and perivascular smooth muscle cells (Figs. 11E, 13B). ER\(\beta\)-ir was only rarely seen in astrocytes, but was observed to be present in some glia cells and oligodendrocytes of, e.g., the anterior commissure (AC). Interestingly, a striking ER\(\beta\)-ir (which could already be seen with the naked eye) was observed in (beaded) fibers of the internal capsule (ic), zona incerta (ZI), stria terminalis, and BSTc, with additional strong neuronal “basket-like” stainings, suggestive of the presence of nerve terminal appositions. Neurons with nuclear, cytoplasmic, and neurite staining were also observed in these areas (see Figs. 5B, 7A–D, 9). Occasionally, predominantly cytoplasmic and sometimes basket-like ER\(\beta\)-ir could be observed in the temporal-, entorhinal-cortex, and amygdala in a few subjects in which adjacent areas also contained these areas (cf. Figs. 13, 14).
Sex differences in neuronal ERβ distribution

Nuclear staining.

Going from rostral to caudal through the hypothalamus and adjacent areas, sex differences in ERβ-ir appeared to be present. More nuclear ERβ-ir was found in men as compared to women in neurons of the medial part of the bed nucleus of the stria terminalis (BSTm), the central part of the BST (BSTc), the islands of Calleja (Cal), the sexually dimorphic nucleus of the medial preoptic area (SDN-POA), the diagonal band of Broca (DBB), ventromedial nucleus (VMN), as well as paratenial nucleus (PT) and paraventricular nucleus of the thalamus (PV) (see Table 2, Figs. 3, 8–11, 15).

Women revealed more nuclear ERβ-ir than men in the suprachiasmatic nucleus (SCN), the supraoptic nucleus (SON), the paraventricular nucleus (PVN), the infundibular nucleus (INF), the nucleus tuberalis lateralis (NTL), and the medial mamillary nucleus (MMN). No sex difference in nuclear ERβ-ir was found in the lateral septum (LS), dorsal and ventral periventricular nucleus (see Table 2, Figs. 3, 6, 10–12, 15).

Cytoplasmic staining.

Sex differences in cytoplasmic ERβ-ir appeared to be present, too, with a stronger staining in women found in the NBM, hDBB, BSTl, Cd, SON, BSTp, INF, NTL, LHA, as well as TM (see, e.g., Figs. 5F,H, 7F). In the MPOA and DPe a sex difference with stronger cytoplasmic ERβ-ir in men was found. Occasionally only INAH-2 neurons of INAH-2-4 could be clearly identified by some cytoplasmic ERβ-ir in a few patients, resembling the cytoplasmic ERβ-ir in SDN-POA/INAH-1 neurons as illustrated in Figure 10B. The SCN, DMN, VMN, PV, PT, and mamillary body complex (MBC) produced an equally strong ERβ cytoplasmic labeling in both sexes (for an extensive overview, see Table 2, Fig. 4). Altogether, the receptor distribution data indicate that there may be functional sex differences in ERβ-ir.

Fiber staining.

Beaded ERβ-ir was observed in various specific brain areas (see Fig. 4) such as the BSTc, hDBB, VMN, INF, ic, and ZI. No clear sex differences were observed in beaded fiber or basket-like staining patterns in the ic and ZI (see Tables 2–4; Fig. 4). A particular fiber ERβ-ir was observed in the BST where perikarya were outlined in a “basket-like” way by ERβ-ir (Figs. 5B,9A,E). Similar “basket-like” staining patterns of neurons were also observed in neurons of the DBB, NBM, ic, ZI, or INF (Figs. 7B,E, 8C). The only sex difference in fiber staining was observed in the hDBB (Ch3) where some fiber staining for ERβ-ir was observed in women but not in men (Fig. 4).
Differences in neuronal ERβ distribution in relation to endocrine status

Some brain areas of the subjects with abnormal estrogen levels (T1, S2, and S8; see Table 1) appeared to display clear differences when compared with their genetic match. For the genetic male subjects with relatively high circulating levels of estrogens (T1 and S2) nuclear or cytoplasmic ERβ-ir appeared to be expressed in typical female levels in areas such as the DBB, NBM, BST,
SCN, SON, PVN, Calleja islands (Cal), VMN, and MB (e.g., Figs. 8D,F, 15C). Interestingly, ERβ-ir seemed to be clearly “upregulated” in DMN of T1 (Fig. 12B). In contrast, the ERβ-ir in the SDN, PV, PT, NTL, and TM of subjects T1 and S2 were not clearly different from their genetic match.

The ovariectomized 46-year-old woman (S2) showed almost no nuclear ERβ-ir (e.g., in the DBB, hDBB, SCN, NBM, TM, VMN, LHA, INF) and in most areas no to low cytoplasmic ERβ-ir with only a weak to intermediate strong cytoplasmic ERβ-ir in the PVN and SON (see, e.g., Fig. 12C–E).

DISCUSSION

In 1996, a novel estrogen receptor was discovered and assigned estrogen receptor beta (ERβ) (Kuiper et al., 1996; Mosselman et al., 1996). Since then, a number of studies have investigated the differences in distribution pattern and function from the “classical” estrogen receptor alpha (ERα) in both peripheral organs and the central nervous system (see, e.g., Lubahn et al., 1993; Shugrue and Merchenthaler, 1997; Kuiper et al., 1998; Laflamme et al., 1998; Lemmen et al., 1998; Krege et al., 1998; McEwen, 1999; Hall and McDonnell, 1999; Ishunina et al., 2000; Österlund and Hurd, 2001; Lecce et al., 2001; Kruijver and Swaab, 2002; Kruijver et al., 2002).

The present study systematically describes the rostrocaudal distribution of estrogen receptor beta immunoreactivity (ERβ-ir) in the human hypothalamus and its adjacent areas of young adults. Neither a long postmortem delay (up to 72 hours) nor a long fixation time (up to 100 days) or cause of death appeared to have affected the ERβ-ir. A similar stability of the sex hormone receptor has also been observed for the hypothalamic AR-ir and ERα-ir in the same patients (Fernández-Guasti et al., 2000; Kruijver et al., 2002). ERβ-ir appeared, however, to be influenced by the presence, or absence, of circulating levels of estrogens, as previously also observed for ERα-ir (Kruijver et al., 2002; see below).

The present study shows that ERβ-ir is present in the nucleus, cytoplasm, and cellular processes of neurons, choroid plexus, oligodendrocytes, glia, and vascular cells in the human hypothalamus and adjacent areas. ERβ-ir was observed more frequently in the cytoplasm than in the nucleus, in contrast to the distribution of ERα, which displayed a more robust nuclear expression pattern (see also Table 3; Figs. 5, 6) (Kruijver et al., 2002). Sex differences in neuronal ERβ-ir were found in various hypothalamic and adjacent areas, which seemed to be predominantly based on “activating” rather than “organizing” effects of steroid hormones as appeared from the patients with high or low circulating concentrations in estrogen levels. Similar activating effects have also been observed for ERα (Kruijver et al., 2002).

**ER-ir in different subcellular compartments and cell-types**

Cytoplasmic receptor staining. Estrogens bind to unoccupied estrogen receptors in the cytoplasm followed by translocation of the complexes to the nucleus,
where they function as transcription factors altering gene expression (Kawata, 1995). The ratio between nuclear and cytoplasmic staining may be influenced by circulating hormone levels as discussed for ERs in, e.g., the SON, basal forebrain, and mamillary bodies (Ishunina et al., 2000; Kruijver et al., 2002). In addition, in the human mamillary body a shift from nuclear to predominantly cytoplasmic AR staining was observed in subjects with low circulating androgen levels. In contrast, a predominantly nuclear AR-ir was observed in subjects with high circulating levels of androgens (Kruijver et al., 2001).

Fig. 8. A-B. The sex difference in nuclear ERβ-ir of diagonal band of Broca neurons (DBB) neurons as represented by a man (A) and a woman (B). C: A conspicuous basket-like ERβ-ir in a DBB neuron. Note the clear appositions on the cell membrane and the clear absence of nuclear and cytoplasmic ERβ-ir. Also note the absence of basket-like ERβ-ir in adjacent DBB neurons. D: Female-like strong cytoplasmic ERβ-ir in a genetic man with high estrogen levels due to an adrenal cortex tumor (S2). E: Cytoplasmic and nuclear ERβ-ir in female NBM neurons. Note the strong nuclear ER-ir in an NBM neuron in contrast to its absence in adjacent NBM neurons, which, however, display a stronger cytoplasmic ERβ-ir. F: Female-like ERβ-ir in an NBM neuron of a castrated and estrogen-treated male-to-female transsexual (T1). Scale bar 28 μM in F (applies to A-D, F); 100 μm for E.
Recently, additional mechanisms of action have emerged, indicating that cytoplasmic and/or membranebound estrogen receptors can cross-talk with second messenger pathway systems, such as the cAMP- and MAPK- (mitogen-activated protein kinase) or calcium signaling pathways (Beyer, 1999; Collins and Webb, 1999; Toran-Allerand et al., 1999; Razandi et al., 1999; Clarke et al., 2000; Kelly and Levin, 2001; McEwen et al., 2001). MAPK-kinases (ERK1 and ERK2) are implicated in sex differences in the unstimulated and estrogen-induced cellular activation of signal transduction cascades, regulating cell

ERβ-ir in the BST area. A: Predominantly cytoplasmic and fiber-like ERβ-ir in a male BSTc, yielding a strong ER-ir that can even be seen with the naked eye. Note the absence of this staining pattern in the BSTm which has, in contrast, a stronger nuclear ERβ-ir (black dots; A,B). C: No such nuclear ERβ-ir was present in the female BSTm. E: Basket-like ERβ-ir suggestive for terminal endings on selective BSTc neurons and beaded fibers. F: Nuclear ERβ-ir in male BSTc neurons at a higher magnification. Note the selective absence (arrow) and presence of basket-like ERβ-ir of adjacent BSTc neurons, which appears to be due to nerve terminal ap-positions coming from fibers projecting onto BSTc neurons (E). Note the selective nuclear ER-ir in F. The arrow points to a negative BSTc neuron for nuclear ER-ir. Scale bar 25 μM in F (applies to E,F); 1 mm for A; 250 μM for B,C.

Fig. 9.
growth, and cell survival in both neurons and astrocytes (Toran-Allerand et al., 1999; Zhang et al., 2002b). Consequently, not only differences in nuclear ERs, but also differences in cytoplasmic ERs may have functional consequences as sex differences in cytoplasmic receptor staining may reflect gender-dependent metabolic activity (Kruijver et al., 2002). Cytoplasmic ERα-ir and ERβ-ir have been observed with various antibodies in neurons in different species, e.g., in the adult guinea pig (ERα-ir: Blaustein and Turcotte, 1989; Blaustein and
Olster (1993), the adult rat (ERα-ir: Blaustein, 1992; ERβ-ir: Li et al., 1997; Simonian and Herbison, 1997; Azcoitia et al., 1999; Zhang et al., 2002a), the neonatal rat (ERα-ir and ERβ-ir: Su et al., 2001), the adult domestic pig (ERα-ir: Van Leeuwen et al., 1995), the adult cow (ERα-ir: Van Eerdenburg et al., 2000), the pubertal primate (ERα-ir: Goldsmith et al., 1997), and in the adult human brain (ERα-ir and ERβ-ir: Ishunina et al., 2000; Ishunina and Swaab, 2001; Kruijver and Swaab, 2002; ERα-ir: Donahue et al., 2000; Kruijver et al., 2002;
ERβ-ir: Savaskan et al., 2001; present study). The combination of nuclear and cytoplasmic neuronal ERβ-ir confirms observations reported by other groups (Li et al., 1997; Simonian and Herbison, 1997; Azcoitia et al., 1999; Patrone et al., 1999; Su et al., 2001; van der Eerden et al., 2002), while exclusive nuclear ERβ-ir in neurons was recently reported by Shugrue and Merchenthaler (2001) in rat. Species differences and differences in specificity and sensitivity of the antibodies used and staining procedures employed may underlie these differences (see also discussion by Shugrue and Merchenthaler, 2001).
It should be emphasized that cytoplasmic staining of steroid receptors is not restricted to estrogen receptors, but appears to be a general phenomenon also observed for glucocorticoid, androgen, and progesterone receptors in the rodent and human brain (Blaustein and Olster, 1993; Webster et al., 1994; Wood and Newman, 1993/1999; Puy et al., 1995; Fernández-Guasti et al., 2000; Kruijver et al., 2001; Kruijver and Swaab, 2002; Kaiser et al., 2003a,b).

**Fiber staining.**

Various human studies have shown ERα and ERβ staining not only in the nucleus and cell body but also in processes of neurons in different brain structures (Donahue et al., 2000; Savaskan et al., 2001; Kruijver et al., 2002; see also Tables 2, 3, 4; Figs. 5, 7C,D, 13C,D, 14A,C). A recent study of McEwen et al.
(2001) confirmed at the electron microscopic level the localization of ERα in dendritic spines and axons of rat hippocampal neurons. In addition, ERα, as stained by MC-20, was also observed in neuronal processes of hippocampal neurons isolated from rat fetuses (Clarke et al., 2000).

The presence of ER-ir in fibers (axons and dendrites) confirms similar observations by other investigators (Blaustein and Turcotte, 1989; Blaustein, 1992; Blaustein and Olster, 1993; Li et al., 1997; Wagner et al., 1998; Clarke et al., 2000; Donahue et al., 2000; Milner et al., 2001; Jakab et al., 2001; Zhang et al.,
Fig. 15. A: Presence of clear nuclear ERβ-ir in VMN neurons of a male and its absence in a female (B). C: Note the female-like absence of nuclear ERβ-ir in VMN neurons of the castrated and estrogen-treated male-to-female transsexual (T1). E,F: The MB sex difference in nuclear ER-ir with the lack of such staining in a male (E) in contrast to strong nuclear ERβ-ir in MMN neurons in a female (F). Note the higher magnification of nuclear ERβ-ir in MMN neurons in Figure 12A. Scale bar = 100 μM in F (applies to A, C, E, F).

2002a; Kruijver et al., 2002). The strong fiber staining for ERβ in a number of brain areas (see Table 2, Fig. 4) and the basket-like staining patterns are suggestive for ERβ-ir appositions of axonal/dendritic terminals on neurons (see Figs. 5B, 7B,E, 8C, 9A–C,E).

Neuritic ERs may be implicated in local nongenomic processes such as estrogen-promoted synaptogenesis (Matsumoto and Arai, 1976; McEwen et al., 2001), the maintenance of neuronal electrical sensitivity (Kelly et al., 1978),
growth and arborization of dendrites (Toran-Allerand, 1991), and neurotransmission (Herbison, 1997). This concept was elegantly reviewed by McEwen et al. (2001). According to the proposed different modes of action of steroids, staining in the cytoplasm and (membrane bound) neuronal processes may thus represent not only unoccupied, but also occupied, forms of ERβ that can become effective (see Razandi et al., 1999; Vasudevan et al., 2001; Abraham et al., 2003; Luine et al., 2003).

*Vascular staining.*

Estrogens can protect against cardiovascular disease in premenopausal women, while menopausal women without replacement therapy show an enhanced prevalence of cardiovascular disease (reviewed by, e.g., Mendelsohn and Karas, 1999). The presence of ERα-ir and ERβ-ir in vascular endothelial and smooth muscle cells of arteries and veins as observed in our studies (Figs. 5H, 11E, 13B) confirms observations of others (Karas et al., 1994; Venkov et al., 1996; Kim-Schulze et al., 1996; Taylor and Al-Azzawi, 2000; Lecce et al., 2001; Andersson et al., 2001). These ERs may take part in protective effects of estrogens on blood vessels (see also Lafrati et al., 1997; Mendelsohn and Karas, 1999) and in estrogen’s regulation of brain blood flow by modulating vascular tone through vasodilatation by targeting both endothelial cells and/or vascular smooth muscle cells (see White, 2002). However, hormone replacement therapy (HRT) may also have negative effects on the cardiovascular system, with enhanced risks to develop coronary heart disease and stroke (thrombosis), depending on the presence or absence of, e.g., progestagens in addition to conjugated equine estrogens (Writing Group for the Women’s Health Initiative Investigators, 2002). The development of new HRT-strategies with “maximal” protective and “minimal” negative effects on the cardiovascular, reproductive, and central nervous system (CNS) will therefore be a major challenge for researchers in the future.

*Astrocyte staining.*

Estrogen actions on glial cells have been implicated in aspects of neuroprotection (Garcia-Segura et al., 1999). ERα-ir and ERβ-ir have been observed with various antibodies in astrocytes in, e.g., adult rat (ERβ-ir: Azcoitia et al., 1999), neonatal rat (ERα-ir and ERβ-ir: Su et al., 2001), and human adult brain (ERα-ir: Ishunina et al., 2000; Donahue et al., 2000; Kruijver et al., 2002). Interestingly, contrary to our observations of a strong ERα-ir in astrocytes (Kruijver et al., 2002), ERβ-ir was rarely observed in astrocytes. This suggests that estrogen actions on astroglia is mediated predominantly by ERα in the young adult human hypothalamus.
Comparison of the distribution of ERβ and ERα.

From Tables 3 and 4, both similarities and differences in distribution pattern and staining intensity of ERβ-ir and ERα-ir become apparent. The different staining patterns of ERα-ir and ERβ-ir in the BSTc, SON, TM, LMN, and INF are illustrated in Figures 5 and 6. It is remarkable that the observed sex differences in nuclear ERβ-ir in the SON, PVN, DBB, and VMN are completely opposite to those for nuclear ERα-ir. When nuclear ERβ in men is high, nuclear ERα is low (DBB), whereas the opposite holds true for the SON and PVN, where nuclear ERβ in men is low and nuclear ERα is high (see Table 3). Similar opposite sex differences were observed for cytoplasmic ERα-ir and ERβ-ir in the NBM, BSTm, BSTl, SON, and INF (see Table 4). Earlier we observed opposite pre- and postmenopausal age related changes in nuclear and cytoplasmic ERα-ir and ERβ-ir in the SON of women (Ishunina et al., 2000). The brain area-dependent differences in expression patterns of ERβ and ERα staining may be related to the capacity of ERβ to downregulate ERα-mediated transcriptional activity (Paech et al., 1997; Hall and McDonnell, 1999). The mechanisms involved might be studied in vitro in human postmortem brain tissue in culture (Verwer et al., 2002).

ERβ distribution in different species

The expression of ERβ in the brain has also been studied in a few other mammalian species. The rat was investigated both by immunocytochemistry (ICC) (Li et al., 1997; Shughru and Merchenthaler, 2001; Burlton-Jones and Tuszynski, 2002) and in situ hybridization (ISH) (Laflamme et al., 1998; Shughru et al., 1996), while the male sheep hypothalamus (Hileman et al., 1999) and human forebrain (Österlund et al., 2000) were studied by ISH. The majority of these studies are in agreement with each other. Low ERβ mRNA levels were found in the rat NBM (Shughru et al., 1997), which is consistent with our data showing only low-to-moderate staining in this area. In line with intermediate ISH labeling of cells in the islands of Calleja and horizontal (hDBB) and vertical limbs (vDBB) of the diagonal band of Broca in the female rat brain (Shughru et al., 1997), we observed low to intermediate nuclear or cytoplasmic ERβ-ir in the DBB and hDBB and Islands of Calleja (see Figs. 3, 4, 7F, 8A). The low nuclear ERβ-ir in human DBB neurons confirm ISH data showing low expression of ERβ mRNA in this region (Österlund et al., 2000). In line with the present data in humans, low ERβ expression has also been detected in the monkey (Gundlah et al., 2000) as well as rat medial preoptic area (Laflamme et al., 1998; Li et al., 1997) and periventricular zone (Li et al., 1997; Shughru et al., 1997). However, some ISH studies in the rat (Shughru et al., 1996, 1997) and male sheep hypothalamus (Hileman et al., 1999) demonstrated high levels of ERβ mRNA and an intermediate ERβ expression at the protein level in the medial preoptic area (Shughru and Merchenthaler, 2001). Robust expression of ERβ has been reported in the BST, PVN, and SON in the
rat brain, both by ISH (Shughrue et al., 1996, 1997) and ICC (Li et al., 1997; Shughrue and Merchenthaler, 2001; Blurton-Jones and Tuszynski, 2002) and in the male sheep hypothalamus by ISH (Hileman et al., 1999). In humans we observed low-to-moderate nuclear ERβ-ir in the male BST-area (medial, central, and posterior divisions), whereas in both males and females a conspicuous fiber staining was detected in the central division of the BST (BSTc) (Figs. 5B, 9A,E). Furthermore, we found clear cytoplasmic staining in the PVN and SON, but nuclear ERβ immunolabeling at low levels was detected in these areas only in women (Fig. 10E,F). These findings agree with human ISH data demonstrating low levels of ERβ mRNA in these regions (Österlund et al., 2000). Using ISH, weakly labeled cells were found in the young, middle-aged and old female rat SCN (Shughrue et al., 1997; Wilson et al., 2002), which is in agreement with the nuclear and cytoplasmic ERβ-ir that was recently observed in neurons of neonatal rat SCN (Su et al., 2001) and in neurons of the human SCN (Kruijver and Swaab, 2002; present study; Fig. 10C).

Consistent with the absence of robust nuclear ERβ-ir in the external globus pallidus (EGP) and nucleus caudatus (present study), no hybridization signal was observed in these basal ganglia forebrain regions in the human brain (Österlund et al., 2000). At the tuberal level the VMN and INF showed consistently low ERβ expression in the rat (Li et al., 1997; Laflamme et al., 1998; Shughrue et al., 1996), sheep (Hileman et al., 1999), and human hypothalamus (Österlund et al., 2000; present study). Also, in the DMN and LHA low ERβ mRNA expression was detected in the rat (Shughrue et al., 1997; Laflamme et al., 1998) and sheep brain (Hileman et al., 1999). Furthermore, the lack of ISH signal in the human DMN (Österlund et al., 2000) agrees with the presently observed absence of nuclear and low cytoplasmic staining in this area. The mamillary bodies in predominantly male subjects were shown not to contain ERβ mRNA in the ISH study by Österlund et al. (2000). Their findings correspond very well to the absence of nuclear and low cytoplasmic ERβ staining in the male LMN and MMN of the present study. In contrast to men, we found that young adult women express nuclear ERβ-ir at relatively low levels compared to ERα, but they have a stronger nuclear ERβ-ir in neurons of the MMN (see Figs. 12A, 15E,F; Table 3).

It should be noted that the few discrepancies that were mainly observed in the intensity of ERβ expression in neurons may not only be related to species differences, but also to variability, differences in gender, age, hormonal status, and methodology (e.g., ISH vs. ICC; different antibodies and immunocytochemical procedures as employed by different groups/studies).

Differences in relation to sex and endocrine status

In 1998 the first ERβ sex difference was described in the developing mouse hypothalamus with RT-PCR. Significantly higher ERβ mRNA levels were detected in male POA from E17 until postnatal day (P) 15 (Karolczak and
Beyer, 1998). These data correspond well with the observed higher nuclear and cytoplasmic ERβ-ir in the adult human male SDN-POA reported here. Furthermore, the presence of nuclear ERβ-ir in the PVN of women, but not in men (Table 2), parallels a similar sex difference obtained with RT-PCR data obtained from rhesus macaques (Pau et al., 1998). However, these findings do not appear to match our observation in the human ic, which displayed low cytoplasmic staining in both sexes, whereas RT-PCR analysis in the ic of rhesus macaques revealed ERβ mRNA only in female subjects (Pau et al., 1998). Again, differences between species, hormonal status, and in the various techniques employed might play a role in the few differences among the species observed so far. Interestingly, a recent ISH study by Scott et al. (2000) comparing ERα and ERβ mRNA by gender in the hypothalamus of sheep revealed, similar to our ER observations, a more robust ERα than ERβ mRNA in the BST, POA, VMN, and ARC area, and a higher ERα expression pattern in the female VMN. The more robust ERβ mRNA expression in the ewe VMN versus the ram VMN contrasts, however, with the reversed sex difference in the human VMN, where men display more intense nuclear ERβ-ir (see Fig. 15A,B). Similar to the findings of Scott et al. (2000), we also observed a robust ERβ-ir in the ZI that could even be observed with the naked eye in contrast to the much lower expression of predominantly nuclear ERα, PR and AR in this area (Kruijver and Swaab, unpublished observations).

The present ERβ distribution and sexually dimorphic findings are also in good agreement with recent reports in rat (Greco et al., 2001; Orikasa et al., 2002; Zhang et al., 2002a). Similar nuclear ERβ-ir sex differences in the DBB, SON, MPOA, and BST, with no sex difference in the periventricular nucleus, were observed in the adult rat brain (Zhang et al., 2002a). Orikasa et al. (2002) reported a sex difference in the distribution of ERβ in the rat periventricular nucleus (Pe) rather than in the total number of its ERβ-expressing cells. This latter observation also seems to fit with the lack of an ERβ sex difference in the human Pe, since, in contrast to rat, the structure of the human periventricular nucleus is organized differently and consists of only a small dorsal and ventral periventricular zone along the third ventricle in both sexes (Koutcherov et al., 2002; cf. Kruijver et al., 2002; see also Figs. 3, 4).

Sex differences in relation to endocrine status

The observed differences in ERβ-ir in the three subjects (T1, S2, and S8) with sex-reversed changes in their circulating estrogen levels appeared to offer hints for potential dynamic effects on ERβ-ir. The differences in these subjects suggest that the observed sex differences in ERβ are mainly due to changes in circulating estrogens and are thus based on “activational” rather than on “organizational” effects. Region-dependent up- and downregulatory effects of circulating estrogen levels are, e.g., observed in respectively the SCN, DMN, and VMN of T1 and SCN and VMN of S2 (see, e.g., Figs. 12B, 15C), while
subject S8 showed a strong decrease in nuclear and cytoplasmic ERβ signal in the SCN, PVN, and SON (see Fig. 12C–E). Similar activational down-regulation and upregulation effects by estrogens on, respectively, nuclear and cytoplasmic ERβ-ir were recently shown in rat peripheral dorsal root ganglion (DRG) neurons (Patrone et al., 1999) and within distinct female rat and male mouse forebrain and hypothalamic neurons (Greco et al., 2001; Nomura et al., 2003). Nuclear ERβ staining in the human endometrium has also been shown to fluctuate throughout the menstrual cycle (Lecce et al., 2001).

**ERα versus ERβ**

**ER knockout mice.**

The development of ER knockout mice has provided an animal model to study the involvement of each of the two receptor subtypes in physiology, brain, and behavior (for review, see, e.g., Couse and Korach, 1999; Pfaff et al., 2000). ERα-knockout (αERKO) male and female mice survive to adulthood with normal external phenotypes. However, males have reduced fertility and females are infertile, with hypoplastic uteri and ovaries with no detectable corpora lutea (Lubah et al., 1993). In contrast, mice lacking ERβ (βERKO mice) develop normally with no apparent anatomical or histological abnormalities of the reproductive organs, fertility, or lactation. However, βERKO female mice do have a reduced ovulation efficiency (Krege et al., 1998), while lordosis behavior remains unaffected (Ogawa et al., 1999). αERKO male and female mice display reduced intromissions, ejaculations, lordosis, and maternal behavior, respectively, whereas the display of mounting behavior per se was not crucially affected (Ogawa et al., 1996, 2000; Versinger et al., 1997). There is, however, a significant reduction in the total number of mount attempts per trial in castrated and testosterone supplemented αERKO male and female mice (Versinger et al., 1997). In addition to the gender-dependent changes in reproduction and sexual behavior, sex-specific changes in aggressive behavior have also been observed in αERKO mice, with enhanced aggression and infanticide levels in females and greatly reduced aggression levels in males (Ogawa et al., 1996, 2000). In contrast, all components of sexual behavior were intact in βERKO male and female mice, with normal or enhanced aggression levels in βERKO male mice (Ogawa et al., 1999; Nomura et al., 2002). In male mice lacking both ERs (αβERKO mice), complete abolition of all components of male sexual behaviors and decreased aggression levels were observed (Ogawa et al., 2000). From these ER knockout data, it seems that fertility and aggression are crucially regulated via ERα and "fine-tuned" by ERβ, while the display of sexual behavior requires the functional presence of both receptor subtypes.

**Potential functional implications**

While keeping in mind the above-mentioned “functional” ER studies in mice, human studies indicate that there is hardly any area that is completely
devoid of ERs. In our studies, except for the external globus pallidus, all brain areas examined express both nuclear and cytoplasmic ERα and ERβ, albeit in a different proportion and in a gender-dependent way (see also Tables 3, 4). To discuss some potential functional and clinical implications of the present findings, we will specifically focus on some ER-expressing brain areas and neuropeptide systems which are implicated in the regulation of autonomic function, mood, reproduction, aggression, and cognition.

The SCN.

The SCN communicates through synaptic pathways with various effector systems to orchestrate circadian and seasonal rhythms (Swaab, 1999; Buijs and Kalsbeek, 2001). Sex differences with more intense nuclear ERs were observed in the SCN of women versus men (see Tables 3, 4), whereas more intense ARs were previously observed in the SCN of men (Fernández-Guasti et al., 2000; Kruijver and Swaab, 2002; Kruijver et al., 2002; present study). The presence of ERs in the human SCN allows for a direct and gender-dependent influence of sex hormones on SCN function, such as the circadian regulation of body temperature, the gonadal axis, the adrenal axis, sleep, and mood (Dijk and Czeisler, 1995; Swaab, 1999; Zhou et al., 2001; Kruijver and Swaab, 2002). Such direct estrogen-SCN effects may be the functional mechanism in sex hormone replacement therapy (SHRT), which restores the circadian fluctuation of body temperature, cortisol levels, blood pressure, sleep, and mood in peri- and postmenopausal women (Mercuro et al., 1998; Gudmundsson et al., 1999; Carlson et al., 2000; Prinz et al., 2001; Soares et al., 2001; Kruijver and Swaab, 2002). The sex hormone sensitive human SCN is thus presumed to be able to monitor and orchestrate biological rhythms of, e.g., the gonadal axis (Kruijver and Swaab, 2002; present study), which displays a circadian rhythm in free estrogen levels as was recently observed in healthy normally cycling women (Bao et al., 2003). The fact that the human SCN expresses, e.g., ERα, ERβ, PR, and ARs already during early gestational brain development (i.e., at least from the second trimester of pregnancy onwards; Kruijver and Swaab, unpubl. obs.) provides further support for the idea that the enlarged SCN in homosexual men (Swaab and Hofman, 1990) may be due to an “organizational” interaction between sex hormones and the developing brain. Such organizing actions may, at least in part, contribute to the establishment of brain circuits responsible for sexual orientation (see Kruijver et al., 2001; Rahman and Wilson, 2003; Swaab et al., 2002; Gooren and Kruijver, 2002). This paradigm is further reinforced by the fact that another human hypothalamic area (INAH-3), varies according to sexual orientation (LeVay, 1991; however, see Byne et al., 2001), and is also a sex hormone sensitive brain area by the expression of, e.g., ERα (Kruijver et al., 2002). The presence of ERα, ERβ, and PR in glia cells, oligodendrocytes, and fibers of the anterior commissure (Kruijver and Swaab, unpubl. obs.; present study), which has been reported to be larger in homosexual men than
in heterosexual subjects (Allen and Gorski, 1992; however, see Lasco et al., 2002), may provide further support for the organizational paradigm of sexual orientation (for recent reviews on the human hypothalamus and sexual orientation, see Swaab et al., 2002; Swaab, 2002; Gooren and Kruijver, 2002).

The mPOA.

The mPOA contains the SDN-POA (= SDN-mPOA or INAH-1) and other INAH cell clusters. The mPOA becomes activated during mating (Blaustein and Greco, 2002; Bakker et al., 2002) and has been implicated in certain aspects of male sexual behavior (e.g., copulation and sexual exhaustion) and aggression (DeJonge et al., 1989; Swaab, 1997; Wang et al., 1997; Gregg and Siegel, 2001; Fernández-Guasti et al., 2002). The SDN-POA is sexually dimorphic in the rodent and human brain, with males having a larger volume and neuron number (Gorski et al., 1978; Swaab and Fliers, 1985; Swaab and Hofman, 1988; Hofman and Swaab, 1989). Although the structural sex difference of the anterior-POA in humans is still a controversial point (Allen et al., 1989; LeVay, 1991; Byne et al., 2000, 2001; for review, see Swaab et al., 2001, 2002; Swaab, 2002; Allen and Gorski, 2002; Gooren and Kruijver, 2002), the stronger AR-ir (Fernández-Guasti et al., 2000) and ERα-ir and ERβ-ir in the SDN-POA in men (Kruijver et al., 2002; present study) is consistent with its reported structural sexual dimorphism. Whether or not the age-dependent sex difference in the SDN-POA volume and neuron number (Swaab et al., 2002; Kruijver et al., unpubl. obs.) and its stronger nuclear ERβ-expression in young adult men (present study) is related to ERβ’s potential role in the prevention of neuronal cell death (Wang et al., 2001), awaits future investigations. From a functional point of view, it is conceivable, e.g., that the sexually dimorphic presence of ERs and both ERβs in the human SDN-POA points to the possible involvement of the human mPOA in gender-dependent aspects of sexual behavior and aggression, as has been reported for animals (Ogawa et al., 2000; Wang et al., 1997; Gregg and Siegel, 2001; Bakker et al., 2002; Blaustein and Greco, 2002; Fernández-Guasti et al., 2002).

The BST.

The human BSTc is sexually dimorphic in size and neuron number (Zhou et al., 1995; Kruijver et al., 2000; Chung et al., 2002). Both measures are larger in males. No relationship between these BSTc measurements and sexual orientation was found, whereas a striking relationship with gender identity was observed. Male-to-female transsexuals had a BSTc with a female size and neuron number, regardless of sexual orientation or adult endocrine status. Excitingly, the reversed pattern was found in the only available brain so far studied of a female-to-male transsexual (Kruijver et al., 2000). The functional implications of these sexually dimorphic findings are still far from clear, but it is important to note that in animals subdivisions of the BST have been implicated in the
regulation of, e.g., female reproductive (lordosis) and maternal behavior (Segovia and Guillamón, 1993; Sheehan and Numan, 2002). The BSTc expresses both ARs and ERs (Fernández-Guasti et al., 2000; Kruijver et al., 2002; Chung et al., unpubl. obs.; present study) with more nuclear ERβ expression of low intensity in young adult men (present study), which may be related to neuronal survival (Wang et al., 2001) and the fact that men have more BSTc neurons than women (Kruijver et al., 2000; Chung et al., 2002).

A conspicuous ERβ-ir of BSTc fibers was additionally observed both in men and women (Figs. 4, 5B, 9; Table 2), which contrasted with the lack of such staining in the BSTm or BSTi (Figs. 4, 9; Table 2). The basket-like ERβ-ir of BSTc neurons (Figs. 5b, 9E) has a striking resemblance to calcitonin gene-related peptide-like pericellular immunoreactivity, which is a marker for putative visceral sensory information onto BST neurons (De Lacalle and Saper, 2000). Together, these data indicate that different subdivisions of the human BST may be functionally different. Furthermore, the presence of ARs, ERs, and progesterone receptors in the adult and developing BSTc (Fernández-Guasti et al., 2000; Kruijver et al., 2002; Kruijver and Chung, unpubl. obs.; present study) support the idea that an altered interaction between sex hormones and the developing brain could have led, at least in part, to the BSTc’s sex reversal in size and neuron number as well as to the reversed gender identity in transsexuals (Zhou et al., 1995; Kruijver et al., 2000).

The SON and PVN.

The SON and PVN produce vasopressin (AVP or ADH) and oxytocin (OXT), which regulate water balance, blood pressure, reproduction, labor and the hypothalamo-pituitary-adrenal (HPA) axis (see Raadsheer et al., 1994; Swaab, 1997; Ishunina et al., 2000; Keck and Holsboer, 2001; Keck et al., 2002; Goncharuk et al., 2002). In rodents, the CRH-producing PVN cells are inhibited by AVP neurons from the SCN (Buijs et al., 1993ab; Kalsbeek et al., 1996). In the human SCN circadian and circannual fluctuations (amplitudes) of neuronal AVP expression levels diminish with aging, while the HPA-axis activity becomes more strongly activated in aged women than in men (Swaab et al., 1994; Hofman and Swaab, 1994, 1995; Seeman et al., 2001). Sex hormone replacement therapy inhibits the enhanced circadian HPA-axis activity in postmenopausal women (Gudmundson et al., 1999; Prinz et al., 2001), indicating a direct or indirect effect on estrogen-sensitive PVN and SCN neurons, respectively (Kruijver and Swaab, 2002; present study). An inhibiting effect of estrogens on PVN CRH neurons could be mediated via nuclear ERβ, while a stimulating effect of estrogens on CRH expression in PVN neurons could be mediated via nuclear ERα. This idea is supported by the fact that nuclear ERα increases and nuclear ERβ decreases in PVN neurons of postmenopausal women (Kruijver and Swaab, unpubl. obs.), while the reversed pattern is observed in young women (Kruijver et al., 2002; present study; Table 3). The observation
that higher estrogen levels are paralleled by stronger nuclear ERβ-ir in PVN neurons may explain estrogen’s inhibiting actions on CRH expression in premenopausal women and would also fit with the reported antidepressant effects by SHRT in postmenopausal women (Sherwin and Gelfand, 1985; Halbreich, 1997). It could also be inferred that a decrease in circulating sex hormone levels during aging may contribute to the enhanced CRH activity in the PVN and the reduced AVP activity in the SCN in elderly with major depression (Raadsheer et al., 1994; Zhou et al., 2001).

The zona incerta.

The zona incerta (ZI) (see Figs. 3, 4) can be considered the most rostral extension of the mesencephalic reticular formation and contains GABA as a major neurotransmitter (Ma et al., 1997) and benzodiazepine binding sites (Najimi et al., 2001). The rostral ZI is continuous with the lateral hypothalamic area and has been implicated in nociceptive and somatosensory perception, locomotion, sexual behavior, feeding and drinking, arousal, and attention (Brown and Grossman, 1980; Edwards and Isaacs, 1991; Heeb and Yahr, 1996; Greco et al., 1996; Ongur and Price, 1998; Iqbal et al., 2001; Mungarndee et al., 2002; Nandi et al., 2002). In addition to ERβ’s presence in the LS, BST, Cal, SDN-POA, PVN, SON, Pe, SCN, NBMC, PV/PT, VMN, TM, INF, MBC, temporal-, entorhinal-cortex, and amygdala, the presence of ERβ-ir in both neurons and fibers of the human ZI, as reported in the present study (Fig. 7A,B), may further underline the potential role of ERβ in sexual behavior, cognition, mood, and homeostatic survival systems. This idea seems to be supported by the fact that in rat reciprocal connections have been reported between the ZI and brain areas that are involved in the regulation of these functions, such as the cerebral cortex, hypothalamus, basal ganglia, basal forebrain, brain stem, and spinal cord (Kolmac et al., 1998; Cheung et al., 1998).

As indicated above, the ZI is an important area in the regulation of food intake and energy metabolism (Dalton and Grossman, 1982; Iqbal et al., 2001; Mungarndee et al., 2002). Interestingly, ERβ was recently shown to mediate the anorectic action of estrogen in rat (Liang et al., 2002). In postmenopausal women, the incidence of obesity increases (Svendsen et al., 1995; Tchernof et al., 2000), while estrogen replacement therapy in postmenopausal women was shown to inhibit increase in body weight and fat accumulation (Tchernof et al., 2000). The balance between ERα and ERβ (isoforms) and their expression patterns in both autonomic central and peripheral systems that are involved in sex-dependent fat metabolism may play a crucial physiological role in this phenomenon (see Rosenkranz et al., 1998; Blaak, 2001; Heine et al., 2000; Cooke et al., 2001; Pedersen et al., 2001; Ohlsson et al., 2000; Kreier et al., 2002). Whether or not site-specific decreases or increases of ERβ in the autonomic part of the CNS (including the zona incerta and specific hypothalamic areas like the INF, see Goldstone et al., 2002) may play an important pathophysiological
role in the development of obesity or the cachectic state of energy expenditure in various diseases (such as anorexia nervosa, AIDS, and Alzheimer's disease) awaits future investigations.

**ERs in the NBMC, TM, and MBC in relation to cognition, mood, and neuroprotection.**

In addition to ERβ mRNA presence in the hippocampus, amygdala, and (entorhinal) cortex (Österlund and Hurd, 2001), the presence of ERβ-ir in neurons of these and other brain areas processing memory, mood, and cognition such as the nucleus basalis of Meynert complex (NBMC), tuberomammillary nucleus (TM), and mamillary body complex (MBC) (present study; see Figs. 5F,H, 7E,F, 12A, 13C, 14B,D, 15E,F) suggests the involvement of ERβ in cognitive and emotional processes, as previously also proposed for AR-ir and ERα-ir in these areas (Tables 3, 4; Fernández-Guasti et al., 2000; Kruijver et al., 2001; Ishunina and Swaab, 2001; Kruijver et al., 2002). Interestingly, other knockout mouse studies implicate ERα to be involved in cognition and neuroprotection (Fugger et al., 2000; Dubal et al., 2001), while ERβ appears also to be involved in adult neuronal survival and neurodevelopment (i.e., ERβ may prevent cell death and/or stimulate neurogenesis and neuronal migration [Wang et al., 2001, 2003; for ERβ-ir distribution in the rat brain, see Shughru and Merchenthaler [2001]). ERβ knockout mice were additionally reported to display higher anxiety levels and reduced levels of activity in open field tests (Krezel et al., 2001), which underscores a potential inhibitory role of ERβ in the regulation of anxiety/mood.

The cholinergic and histaminergic systems in the brain display circadian expression rhythms with higher expression levels during wakefulness (Kametani and Kawamura, 1991; Tuomisto and Tuomisto, 1982; Prell et al., 1989; Kiviranta et al., 1994; Brown et al., 2001), which is in agreement with involvement of both systems in arousal and alertness (Mallick and Joseph, 1997; McEwen and Alves, 1999; Passani et al., 2000; Scammell et al., 2000). The presumed crucial role of acetylcholine and histamine in cognition and alertness is further underlined by the degeneration and decreased activity levels of both systems in Alzheimer's disease (AD) (Mazurkiewicz-Kwilecki and Nsonwah, 1989; Salehi et al., 1995, 1998; Swaab et al., 1998; Gibbs and Aggarwal, 1998; van Leeuwen et al., 1998, 2000; Kim et al., 2001). Regarding the sex hormone sensitivity of the human cholinergic NBMC system in health and disease (Fernández-Guasti et al., 2000; Ishunina and Swaab, 2001; Kruijver et al., 2002; present study), it should be noted that the ER-expressing neurons in the thalamic PT and PV (Kruijver et al., 2002; present study; Fig. 11E,F) are also cholinergic (Jones, 1997) and have, in addition to the SCN, also been implicated in the modulation of circadian rhythms and mood (Moga et al., 1995; Ji et al., 1998).
Neuroprotection by SHRT.

Postmenopausal estrogen replacement therapy was found to delay the onset of AD, lower the risk of disease, and improve mood and cognition (Tang et al., 1996; Asthana et al., 1999, 2001; Halbreich, 1997; Wise, 2002). Lower endogenous free and total serum estradiol levels are also correlated with poor cognitive performance in elderly with and without AD (Yaffe et al., 2000; Senanarong et al., 2002). Whether the central changes in ERα/β-ratios in AD (Ishunina and Swaab, 2001; Savaskan et al., 2001; Ishunina et al., 2002; Hu et al., 2003) are due to decreased endogenous estrogen levels, enhanced HPA-axis activity, dynamic changes in ER function, local changes in aromatase function, or neurosteroid metabolism remains to be elucidated.

SHRT has recently also been shown to have potential beneficial effects in the treatment of Parkinson’s disease, major depression, and schizophrenia (Blanchet et al., 1999; Tsang et al., 2000; Schneider et al., 1997; Amsterdam et al., 1999; Kornstein et al., 2000; Seeman and Lang, 1990; Kulkarni et al., 2001; Lindamer et al., 2001; Garcia-Segura et al., 2001; Stevens, 2002). Whether or not SHRT restores changes in ERα/β ratios and/or improves metabolic activity in the affected brain areas awaits more investigations on the neuroprotective mechanisms of SHRT.

CONCLUSIONS

Almost all brain areas examined expressed both ERα (Kruijver et al., 2002) and ERβ (present study), albeit in a distinct and gender-dependent way, with ERα showing a more robust nuclear expression pattern than ERβ. Sex differences in nuclear and cytoplasmic ERβ-ir were found in the young adult human hypothalamus and adjacent areas, which, analogous to ERα, appear to be due to “activation” rather than “organizational” effects of circulating estrogens.

From our previous and present estrogen receptor findings it can be inferred that area-dependent dynamic changes in ERα and ERβ expression (ratios) are likely to play a role in sex-dependent changes in a multitude of central functions in health and neuropsychiatric diseases.

LITERATURE CITED


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