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Differential Expression of Estrogen Receptor α and β Immunoreactivity in the Human Supraoptic Nucleus in Relation to Sex and Aging*

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ABSTRACT

The dorsolateral supraoptic nucleus (dl-SON) is the main production site of plasma arginine vasopressin (AVP). Plasma AVP levels and the activity of AVP neurons in humans are higher in males than in premenopausal females. On the other hand, an increased activity of AVP neurons becomes prominent in postmenopausal women who have strongly decreased estrogen levels. As estrogens are presumed to inhibit AVP production in a receptor-mediated way, we studied estrogen receptor (ER) α and β immunoreactivity in the dl-SON. Hypothalami of 34 controls were subdivided into 4 groups within a 50-yr boundary (young men, young women, elderly men, and elderly women). The AVP part of the dl-SON of young women contained 50 times more neurons with ERβ nuclear staining than that in young men and 250 times more than that in elderly women. In addition, young women also showed more ERβ cytoplasmic staining than young men and elderly women. In contrast to the ERβ immunoreactivity, no differences were found in the number of ERα-positive neurons in the 4 groups, but the age and sex pattern of ERα staining was basically opposite that of ERβ. Significant correlations between the percentage of ERβ- and ERα-positive and -negative AVP neurons and age were found in women, but not in men. Our data demonstrate for the first time a strong decrease of ERβ and an increase of ERα immunoreactivity in AVP neurons of the dl-SON of postmenopausal women. Both receptor changes are proposed to participate in the activation of the AVP neurons in postmenopausal women. (*J Clin Endocrinol Metab* **85:** 3283–3291, 2000)

THE SUPRAOPTIC nucleus (SON) is the main production site of plasma arginine vasopressin (AVP), a neurohormone that is involved in water balance, electrolyte, and blood pressure regulation and has also central effects (1). It was shown in humans that plasma AVP levels are higher in males than in females (2, 3). Sex hormones may be involved in this sex difference, as plasma AVP levels change during the menstrual cycle (4, 5) and after administration of oral contraceptives (5). Recently, we observed age-related sex differences in AVP neuronal activity in the human SON. AVP neurons were more active in young men than in young women (≤50 yr of age), whereas these neurons appeared to become activated in postmenopausal women (6, 7). Our data suggest an inhibitory role of estrogens in the activity of AVP neurons in the human SON, and we presumed that these changes were estrogen receptor (ER) mediated. ERs were shown to be abundantly expressed in the SON in the rat (8, 9), the ewe (10), and the monkey (11, 12). To date two genomic subtypes of ER have been cloned in humans and rodents: ERα (13) and ERβ (14). They play different roles in gene regulation; ERα activates and ERβ inhibits transcription in HeLa cells (15). Interestingly, the primate hypothalamus contains more ERβ messenger ribonucleic acid (mRNA) than ERα (16). In the rat SON, ERβ mRNA, but not ERα mRNA, was found (9), and AVP cells were demonstrated to colocalize ERβ and ERβ mRNA (17, 18). To date, no information is available on the presence of ERα and ERβ in the human hypothalamus. The aim of the present study was to analyze ERβ and ERα expression in the AVP neurons of the human SON in relation to age and sex. We studied groups of subjects subdivided within a 50-yr boundary, which is the mean age of the menopause (19), to find out whether there was a sex difference in ER immunoreactivity in AVP neurons and a menopause-associated ER decrease in women. Such changes are of particular interest, because prominent sex and age differences in the incidence of hypertension and cardiovascular diseases have been reported. Premenopausal women have a 3–4 times lower prevalence of hypertension as age-matched men, whereas in women after the menopause this sex difference is reversed (20, 21). All measurements were performed in the dorsolateral part of the SON (dl-SON), in which 90–95% cells are vasopressinergic (1, 22). In the present study the small number of oxytocin (OT) neurons that are localized preferentially in the cap of the dl-SON (1) was identified on the basis of OT-stained adjacent sections, and only a semiquantitative analysis of ERs immunoreactivity was performed in this small group of neurons.

Materials and Methods

Tissue collection

Brains from 34 control subjects (16 males and 18 females) without a primary neurological or psychiatric disease and ranging in age from

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20–94 yr (mean ± SEM, 54.9 ± 3.5) were obtained at autopsy (see Table 1 for clinicopathological information). The hypothalami were dissected and fixed in 4% formaldehyde in phosphate-buffered saline (pH 7.4) at room temperature, for about 2 months. The fixed hypothalami were dehydrated in graded ethanols, embedded in paraffin, and cut serially in 6-μm coronal sections. For anatomical orientation, every 50th section was mounted on chrome-aluminum sulfate-coated glass slides, deparaffinized, hydrated, and stained with thionine (0.5%). Two adjacent sections per patient in the middle of the dl-SON were mounted for immunocytochemistry.

Immunocytochemistry

Immunocytochemical detection of ERβ was performed as follows: deparaffinization in xylene and graded ethanols, rinsing in distilled water twice for 5 min each time, rinsing in Tris-containing buffered saline (TBS)-high salt (pH 7.6) twice for 5 min each time, microwave pretreatment in 0.1 mol/L citrate buffer (pH 6.0) twice for 5 min each time at 700 watts, washing in TBS-high salt twice for 5 min each time, incubation in milk-TBS for 1 h at room temperature, washing in TBS-high salt twice for 10 min each time, and incubation with a primary polyclonal goat anti-ERβ antibody corresponding to an amino acid sequence mapping at the amino-terminus of ERβ of human origin (catalogue no. sc-6820, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted 1:50 in Supermix (0.25% gelatin and 0.5% Triton X-100 in TBS, pH 7.6) for 1 h at room temperature and at 4 C overnight. The next day the sections were washed in milk-TBS twice for 10 min each time; washed in TBS-high salt twice for 5 min each time; incubated with secondary biotinylated antigoat IgG (Vector Laboratories, Inc., Burlingame, CA) 1:200 in Supermix for 1 h at room temperature; washed in milk-TBS twice for 10 min each time; washed in TBS-high salt for 10 min; incubated with avidin-biotin complex (ABC, Elite kit, Vector Laboratories, Inc.) 1:800 in Supermix for 1 h at room temperature; washed in TBS-high salt twice for 10 min each time; incubated with biotinylated tyramine diluted 1:500 in TBS plus 0.01% hydrogen peroxide (H_2O_2) for 15 min at room temperature; washed in TBS-high salt twice for 10 min each time; incubated with ABC complex as described above; rinsed in 0.05 mol/L Tris-HCl (pH 7.6); incubated in Tris-HCl containing 0.05 mg/ml 3,3′-diaminobenzidine (Sigma, St. Louis, MO), 0.01% H_2O_2, and 0.2% nickel ammonium sulfate; washed in Tris-HCl twice for 10 min each time; dehydrated in graded ethanols; cleared in xylene; and coverslipped with Entellan mounting medium (Merck & Co., Darmstadt, Germany).

ERα staining was performed as follows: deparaffinization in xylene and graded ethanols, rinsing in distilled water twice for 5 min each time, rinsing in TBS (pH 7.6) twice for 5 min each time, water bath pretreatment in 0.05 mol/L Tris-HCl buffer (pH 7.6) for 30 min at 90 C, washing in TBS twice for 5 min each time, incubation in milk-TBS for 1 h at room temperature, washing in TBS for 5 min, and incubation with a primary polyclonal rabbit anti-ERα antibody recognizing the carboxyl-terminus epitope of the ERα (Santa Cruz Biotechnology, Inc., catalogue no. sc-542) diluted 1:100 in Supermix-milk (0.25% gelatin, 0.5% Triton X-100, and 5% milk powder in TBS, pH 7.6) for 1 h at room temperature and at 4 C overnight. The next day the sections were washed in milk-TBS three times for 10 min each time; washed in TBS for 5 min; incubated with secondary biotinylated antirabbit IgG (Vector Laboratories, Inc.) 1:200 in Supermix-milk for 1 h at room temperature; washed in TBS three times for 10 min each time; incubated with ABC (Elite kit, Vector Laboratories, Inc.) 1:800 in Supermix for 1 h at room temperature; rinsed in 0.05 mol/L Tris-HCl (pH 7.6); incubated in Tris-HCl containing 0.05 mg/ml 3,3′-diaminobenzidine (Sigma), 0.01% H_2O_2, and 0.2% nickel ammonium sulfate; washed in Tris-HCl twice for 10 min each time; dehydrated in graded ethanols; cleared in xylene; and coverslipped with Entellan mounting medium (Merck & Co.).

To localize the area containing OT neurons at the dorsal side of the SON (1), adjacent sections were stained with a monoclonal mouse antibody (A1–28). Briefly, after rehydration sections were incubated with A1–28 (1:200), biotinylated horse antimouse IgG (1:200; Vector Laboratories, Inc.), ABC complex (1:800), and 0.05 mol/L Tris-HCl containing 0.05 mg/ml 3,3′-diaminobenzidine, 0.01% H_2O_2, and 0.2% nickel ammonium sulfate.

The intensity of the staining was estimated semiquantitatively at light microscopy according to the following scale: +++, strong; ++, moderate; +, weak; + −, very weak; and −, absent (see Table 1).

Specificity of the antibody

According to the Santa Cruz Biotechnology, Inc., catalog, the ERβ antibody is specific for ERβ and does not cross-react with ERα, and the ERα antibody is specific for ERα and does not cross-react with ERβ. We confirmed the specificity of these antibodies in the following experiments. 1) In a spot blot test the antibodies were shown to recognize the blocking peptides, whereas 2) an adsorption test resulted in the blocking of the antibodies with the peptide and elimination of the staining. 3) Staining of adjacent sections with the antibody against the C-terminus of the ERβ (Santa Cruz Biotechnology, Inc., catalogue no. 6822) (23) revealed the same pattern as with the antibody against the N-terminus of the ERβ used in the present study. 4) Human ovary and testis samples were stained in alternating sections, because in both organs the two ER subtypes (ERβ and ERα) are known to be expressed (23). In the ovary, ERβ cytoplasmic staining was observed in granulosa cells and follicles, which is consistent with a recent study in the rat (24), whereas both nuclear and cytoplasmic staining were found in the adipose and connective tissues. In the testis, Leydig and connective tissue cells showed nuclear ERβ staining, whereas weak nuclear and cytoplasmic staining was observed in Sertoli cells and spermatocytes. In the pituitary, mainly weak cytoplasmic ERβ staining was present. Interestingly, staining with an ERα antibody revealed a different pattern of staining not only in the hypothalamus (Kruijver, F. P. M., *et al.*, in preparation), but also in the pituitary, testis, ovary, and uterine tube. In the ovary, follicles and stroma cells were stained more intensively with anti-ERα. Also, secretory cells of the Fallopian tube showed nuclear staining, which was absent in ERβ sections. In testis, Leydig cells showed weaker nuclear and cytoplasmic staining for ERα compared with ERβ, whereas no staining was observed in connective tissue cells. Sertoli cells and some spermatocytes demonstrated cytoplasmic and nuclear stainings, which were stronger than those for ERβ. In the pituitary, with anti-ERα clear nuclear and more intense cytoplasmic staining was observed, whereas only weak cytoplasmic staining was present in the ERβ-stained pituitary, which is in concordance with the study in the rat pituitary, where ERβ was expressed at a lower level than ERα (25). 5) The present study supports the specific staining of ERβ and ERα, as a different pattern of staining was found for the two receptors in the SON in relation to age and sex. 6) Recently, Western blot analysis was successfully performed (24) for the ERα antibody that was used in the present study. 7) Staining without primary antibody produced absolutely no staining. Taken together these results demonstrate the specificity of the antibody used.

Image analysis

As 90–95% of SON cells are vasopressinergic (1), and the small amount of OT cells could be distinguished on the basis of their dorsal localization in adjacent sections stained for OT and their cytoarchitectonic characteristics, AVP neurons were the main focus in the present study. We analyzed the number of AVP cells that contained a nucleolus, with nuclear or cytoplasmic ERβ or ERα staining as well as the number of neurons negative for ERβ or ERα using an IBAS image analysis system (Kontron Instruments Ltd., Zurich, Switzerland; KAT-based system) (6). The image analysis system was connected to a Sony XC-77CE black and white CCD camera (Tokyo, Japan) equipped with a chalnycon tube mounted on a Carl Zeiss microscope (New York, NY). All measurements were performed using a 560-nm pore size filter, which coincides with the maximum absorption of the diaminobenzidine/nickel sulfate precipitate in the sections. Area selection was performed as follows. In each section to be analyzed, an area covering the SON (using the ×2.5 objective of the microscope) was loaded into the IBAS and displayed on the image analysis monitor. The position of the section under the microscope was stored using the *x-y-z* coordinates of the scanning stage. In this image the contour of the dl-SON was outlined manually. To select a number of fields in the SON, a grid that consisted of areas corresponding to the image size at a ×500 magnification (×40 objective) was superimposed automatically over the SON area. From this grid all fields were automatically selected. The position of each microscopic field belonging to this sample was again expressed in the *x-y-z* coordinates of the scanning stage. On the basis of these coordinates the fields were re-

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TABLE 1. Clinico-pathological information on subjects and the intensity of ERβ and ERα staining in the SON

NBB no.	Age	Sex	Bw (g)	Pmd (h)	Fix (d)	AVP (ERβ)		OT (ERβ)		AVP (ERα)		Cause of death
						N+	C+	N+	C+	N+	C+	
1) 94040	20	m	1490	8.00	82	−	+	+++	+++	+	++	Heart failure and acute fibrinous haemorrhagic pneumonia, B cell non-Hodgkin lymphoma
2) 85041	28	f	nr	5.4	44	+++	++	−	+++	−	+	Cardiogenic shock
3) 85027	29	f	1150	13.17	60	−	+++	−	++	++	+++	Coma hepatica
4) 88017	31	m	1550	96.00	24	−	+	−	+−	−	+	Cardiac failure due to coronary anomaly and acute infarction
5) 91005	31	m	1377	34.00	35	−	+	−	+−	+++	+++	Carcinoma of the adrenal gland, metastases, cardiac failure
6) 92037	32	f	1280	30.00	45	+−	+++	−	+++	−	+	Bronchopneumonia, lung edema
7) 84026	33	f	1250	22.75	65	+−	+++	−	++	−	+	Asthmatic bronchitis, lung oedema, hypoxia, coma
8) 97075	33	m	1410	18.75	32	−	++	−	++	−	+++	Traffic accident
9) 97082	36	m	1628	28.66	42	−	++	−	++	++	+	Choriocarcinoma of the testis with extensive metastases
10) 97101	43	m	1380	9.25	130	−	++	−	+++	+	++	Pneumonia
11) 80002	46	f	1300	2.5	36	−	+++	−	+++	+++	++	Ovarian carcinoma, bilateral ovariectomy, metastases, sepsis
12) 89104	49	f	1260	41.00	33	+	++	−	++	+	+	Lung carcinoma, shock kidney
13) 94118	49	m	1254	22.33	33	−	+	−	+−	−	+	Sepsis
14) 96423	49	f	1253	17.00	806	++	++	−	+++	−	+	Lung tumor with metastases, massive thromboemboly
15) 93072	50	m	1573	9.00	52	+	++	−	+−	+++	+++	Carcinoma of the bladder with metastases, sepsis, lung edema
16) 97108	50	f	1219	43.2	31	+	+++	−	+++	−	++	Pneumonia, septic shock
17) 86039	53	f	1410	27.00	17	−	+−	−	+−	−	+	Chronic myelocytic leukemia, blastomatosis
18) 92047	53	m	1410	14.00	31	++	++	+−	+++	−	++	No cerebral abnormalities, cause of death unknown
19) 92046	54	f	1080	12.75	396	−	+−	−	+	−	+	Traffic accident
20) 95007	54	m	1335	9.17	34	−	++	−	+	+	++	Bleeding from the right artery carotis communicans
21) 96014	54	f	1257	8.00	28	−	+	−	+	++	+	Acute renal failure, mammacarcinoma, metastases
22) 97103	55	m	1530	41.75	70	−	+	−	+++	−	+	Lung tumor, pericarditis
23) 94114	63	m	1154	26.75	29	−	+	−	+	+	+++	Sigmoidectomy, postoperative complications, pneumonia
24) 96013	68	f	1122	10.5	32	−	+	−	+	++	+	Hematemesis and melena, breast carcinoma, metastases
25) 96057	69	f	1074	8.5	31	−	++	−	+	++	++	Myocardial infarction, lung emboli, hemorrhage
26) 93073	73	f	1344	8.00	34	−	++	−	+	++	++	Bronchopneumonia, sepsis
27) 95106	74	m	1317	8.00	60	−	++	−	+	−	++	Myocardial infarction, cardiac failure
28) 93139	78	f	1135	6.42	32	−	++	−	+	++	+++	Respiratory insufficiency
29) 94076	78	m	1442	8.42	24	−	+	−	+	+	+	Cardiac arrhythmia
30) 94057	81	f	1350	7.25	31	−	+	−	+	+++	++	Bleeding in the upper abdominal area
31) 90031	82	m	1268	5.33	27	−	+−	−	+−	+	++	Urotheliumcell carcinoma with metastases, peritonitis
32) 94074	85	f	925	5.17	28	−	++	−	+	+	+++	Pneumonia
33) 93012	92	m	1038	12.5	33	−	++	−	+	−	+++	Myocardial infarction, pneumonia
34) 96132	94	f	1118	6.17	29	−	+	−	+	−	+++	Bronchopneumonia, sepsis

NBB, Netherlands Brain Bank; m, male; f, female; Pmd, postmortem delay (in hours); fix, fixation time (in days); Bw, brain weight (in grams); nr, not recorded; N+, nuclear positive; C+, cytoplasm positive; AVP, arginine vasopressin neurons; OT, oxytocin neurons.

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trieved for measurement. To determine the number of cells displaying nuclear, cytoplasmic, or negative ERβ and ERα staining; the ×40 objective was positioned in the microscope; and the scanning stage was moved to the previously defined positions of the high magnification measuring areas.

In the present study we defined, in addition to nuclear and cytoplasmic staining, a new category of ERs staining in neurons, *i.e.* perinuclear staining. This is the presence of a thin black band around the nucleus, probably corresponding to the perinuclear band of endoplasmic reticulum found around the nucleus in the neurosecretory neurons of the SON (26). After the image analysis was finished, the percentage of cells with the three different types of staining was calculated.

Statistical methods

The differences in the percentages of cells with the different types of staining between males and females in various age groups were tested using the two-way ANOVA and *t* test. To test the correlation between different parameters, such as fixation time, postmortem delay, age and sex of the subjects, and the mean percentage of ER-positive cells, linear regression analysis was used. $P < 0.05$ was considered to be significant.

Results

The intensity and number of ERβ-stained AVP neurons was higher in young women than in the other three groups, whereas less ERα staining was observed in this group. In contrast, more intensely nuclear and cytoplasmic ERα-stained AVP neurons were observed in men and in elderly women compared to young women. In some patients nucleoli were stained for ERα, but not for ERβ. Muscle and endothelial cells and probably pericytes of blood vessels also showed different patterns of staining with the two antibodies. They were intensely stained for ERβ and less intensely or not at all stained for ERα. Astrocytes were stained for both ERs.

AVP neurons

Only in young women (28–50 yr old) did a large proportion of AVP neurons show both nuclear and cytoplasmic ERβ immunoreactivity (Table 2 and Figs. 1 and 2D). In the other three groups there was only a very small proportion of ERβ-positive neurons. The cells in these three groups showed preferentially cytoplasmic staining for ERβ and hardly any nuclear staining. In young men (Table 1 and Fig. 2B) the majority of neurons were negative for ERβ. Elderly women (>50 yr old; Fig. 3D) showed almost exclusively cytoplasmic staining for ERβ, which was, moreover, weaker and present in fewer neurons than in young women (Table 1). In contrast, in ERα-stained sections the intensity of nuclear staining in AVP neurons was higher in elderly women and young men than in young women (Table 3 and Figs. 2 and 3). The intensity of cytoplasmic staining for ERα was also higher in elderly women and men than in young women (Table 1). More negative cells were found in young women than in any other group studied.

A quantitative study was performed on AVP neurons. On the average, 207 ± 16 (for ERβ) and 320 ± 27 (for ERα) AVP cells/patient were analyzed. Data for the young ovariectomized patient (no. 80002, Table 1) were not included for the analysis.

ERβ

The two-way ANOVA test showed that both sex and age were important for the percentage of AVP neurons staining for ERβ in the nucleus [main effects (ME): $F(2,32) = 5.016$; $P = 0.013$; the interaction effect between sex and age (IE): $F(1,32) = 6.968$; $P = 0.013$] and in the cytoplasm [ME: $F(2,32) = 3.773$; $P = 0.035$; IE: $F(1,32) = 4.134$; $P = 0.051$] or not staining for ERβ [ME: $F(2,32) = 3.773$; $P = 0.035$; IE: $F(1,32) = 4.134$; $P = 0.051$]. The proportion of AVP neurons staining for ERβ in the nucleus was 50 times larger in young women than in young men ($P = 0.019$) and 250 times greater

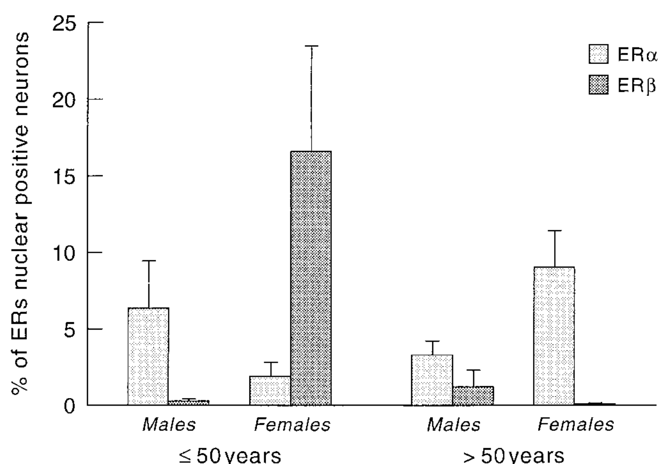

FIG. 1. Graph depicting differential expression of nuclear ERβ and ERα in AVP neurons in the dl-SON in relation to age and sex. In young women (39.5 ± 3.47 yr old; 6 subjects), the percentage of nuclear ERβ-positive neurons is 50 times higher than that in young men (36.63 ± 3.6 yr old; 8 subjects) and 250 times higher than that in elderly women (70.9 ± 4.46 yr old; 10 subjects), whereas the proportion of ERα-positive cells in elderly women and in young and elderly (68.88 ± 5.2 yr old; 8 subjects) men exceeds that in young women 4.5 and 3 times, respectively.

TABLE 2. The mean percentage of ERβ-stained AVP neurons in the SON in different age groups

	Age ≤50 yr		Age >50 yr	
Sex	Male	Female	Male	Female
No.	8	6	8	10
Nucl+ (%)	0.26 ± 0.14[a]	16.6 ± 6.9[a,b]	1.2 ± 1.1	0.07 ± 0.07[b]
Cytopl+ (%)	78.3 ± 5.9[a]	97.9 ± 1.4[a,b]	85.9 ± 4.5	88.8 ± 2.9[b]
Neg− (%)	21.8 ± 5.9[a]	2.1 ± 1.3[a,b]	14.1 ± 4.5	11.2 ± 2.9[b]

Nucl+ (%), The percentage of cells with nuclear staining; Cytopl+ (%), the percentage of cells with cytoplasmic staining; Neg− (%), the percentage of negative cells.

[a] Statistically significant difference between young men and young women.

[b] Statistically significant difference between young women and elderly women.

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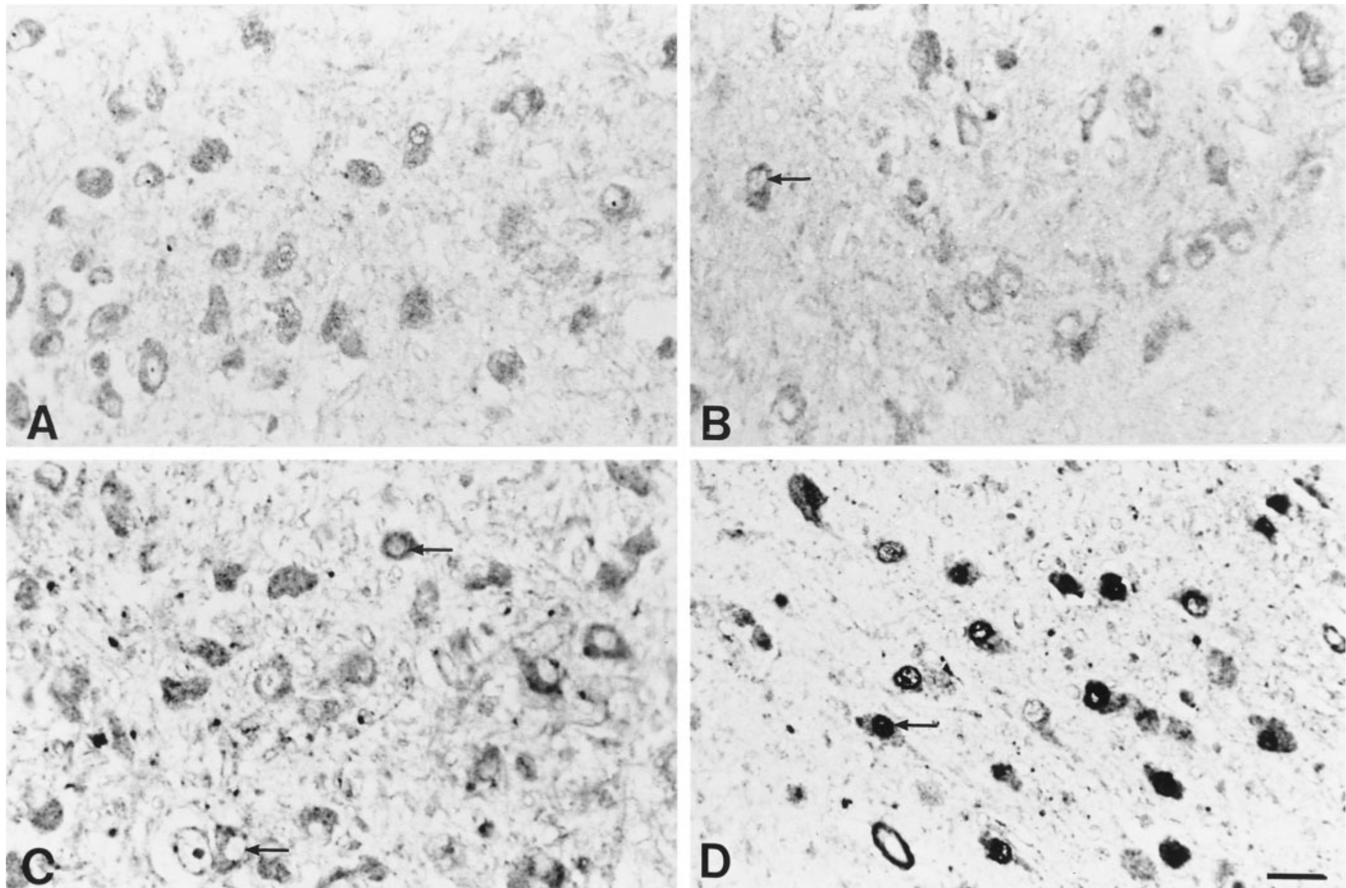

FIG. 2. Immunocytochemical staining of ERα (A and C) and ERβ (B and D) in the dl-SON of young patients. The males are depicted in A and B; the females in C and D. In a young man (B) there is only cytoplasmic staining for ERβ, whereas ERα nuclear staining is present (A). In a young woman AVP cells show ERβ nuclear staining (D) and weak or moderate ERα cytoplasmic staining (C). *Bar*, 42 μm. The *arrowheads* in B and C indicate perinuclear staining; in D they show nuclear staining.

in young than in elderly women ($P = 0.008$; Table 2 and Fig. 1). The percentage of cells expressing cytoplasmic ERβ in young women (97.9%) exceeded that in young men (78.3%; $P = 0.007$) and that in elderly women (88.8%; $P = 0.048$). The proportion of AVP neurons negative for ERβ was the highest in young men (21.8%). In young women this percentage was 10 times lower than in young men ($P = 0.007$) and 5 times lower than in elderly women ($P = 0.048$; Table 2).

The percentage of perinuclear stained neurons was also dependent on age and sex [ME: $F(2,32) = 44.026$; $P < 0.0001$; IE: $F(1,32) = 32.748$; $P < 0.0001$]. It was 7 times higher in young women (54%) than in young men (8%; $P = 0.643$) and in elderly women (8%, $P = 0.107$) and was significantly higher in young men than in elderly men ($P = 0.033$) and in elderly women than in elderly men ($P = 0.05$). The pattern for the four groups followed that of cytoplasmic staining.

Because the subdivision of the groups at the age of 50 yr is arbitrary, we also performed linear regression analysis that demonstrated a correlation between the percentage of nuclear ERβ-positive AVP cells and age ($r = 0.509$; $P = 0.002$) and between the percentage of cytoplasm positive and negative cells and sex ($r = 0.456$; $P = 0.008$). After subdivision into males and females, the correlation between age and the percentage of nuclear ($r = 0.682$; $P = 0.002$), cytoplasmic ($r = 0.571$; $P = 0.013$), and negative ($r = 0.571$; $P = 0.013$) cells was present only in females and was absent in males ($r = 0.288$; $P = 0.279$ and $r = 0.255$; $P = 0.341$, respectively).

ERα

Age and sex had no significant effect on the proportion of ERα-stained neurons (Table 3 and Fig. 1), but a significant IE between these two factors was present for the percentage of nuclear ERα-positive AVP neurons [IE: $F(1,32) = 4.703$; $P = 0.038$]. The proportion of nuclear ERα-positive neurons was higher in young men than in young women ($P = 0.018$) and higher in young men than in elderly men ($P = 0.015$). Linear regression analysis showed a significant relationship between the proportion of cytoplasmic ERα-positive AVP neurons and age ($r = 0.419$; $P = 0.015$) and between the percentage of ERα-negative neurons and age ($r = 0.419$; $P = 0.015$) in the whole group. After subdivision into males and females, the above-mentioned relationships ($r = 0.610$; $P = 0.007$) and the correlation between the percentage of nuclear ERα-positive cells and age ($r = 0.548$; $P = 0.019$) were found again only in women and not in men ($r = 0.348$; $P = 0.204$ and $r = 0.980$; $P = 0.727$, respectively).

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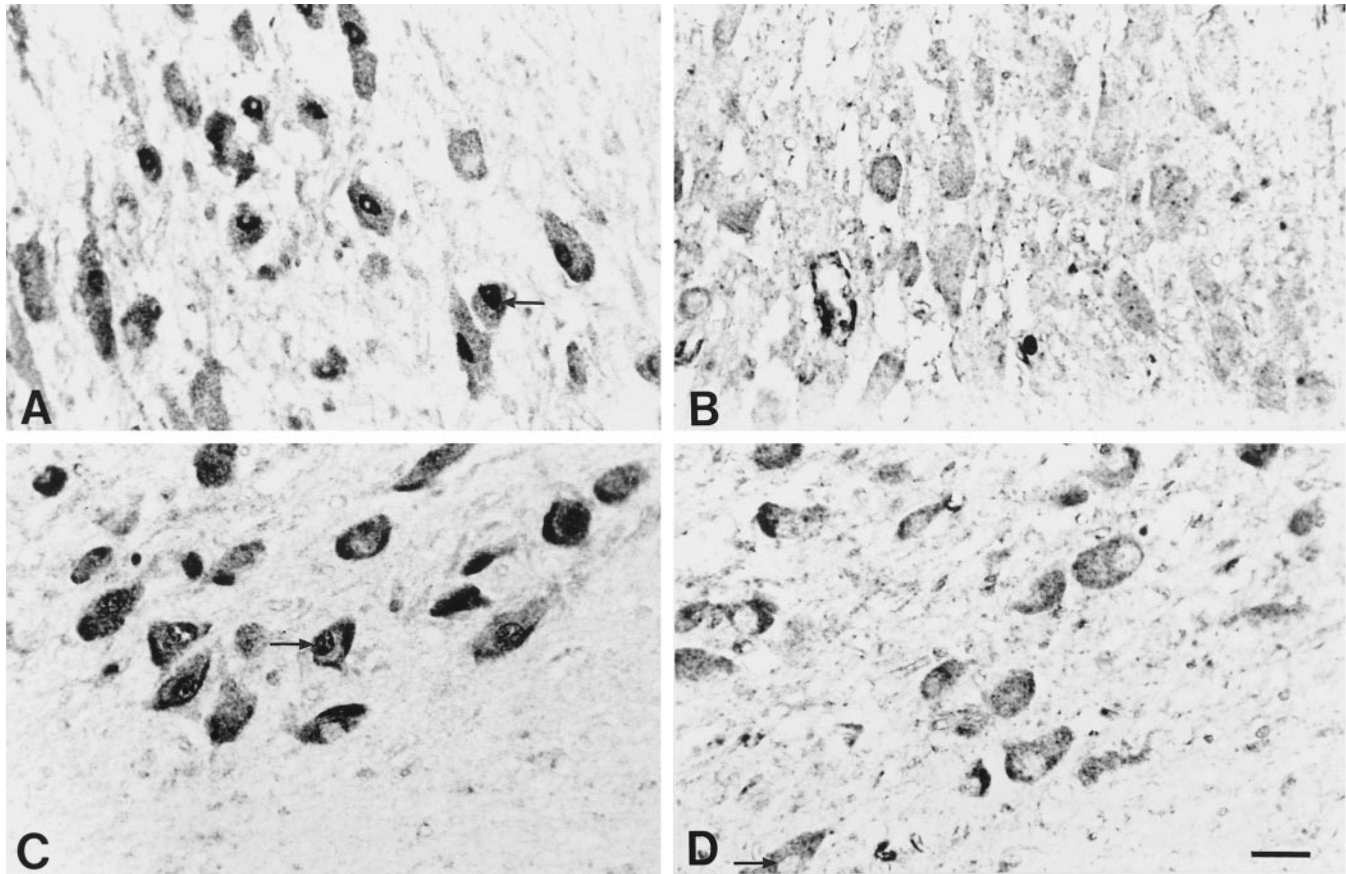

FIG. 3. Immunocytochemical staining of ERα (A and C) and ERβ (B and D) in elderly patients in the dl-SON. The males are depicted in A and B; the females in C and D. Note the weak ERβ cytoplasmic staining (B and D) and strong ERα nuclear staining (A and C) in the AVP cells. In the elderly woman there is moderate ERβ cytoplasmic (D) and prominent ERα nuclear (C) staining. *Bar*, 42 μm. The *arrowheads* in A and C indicate nuclear staining; in D they show perinuclear staining.

OT neurons

OT neurons were identified in the SON on the basis of their position (a small number of cells in the cap of the dl-SON) (1) and their cytoarchitectonic characteristics, *i.e.* smaller size and OT staining in adjacent sections. All subjects demonstrated cytoplasmic staining of OT neurons for ERβ, whereas only a few OT cells showed nuclear staining (patients 94040 and 92047). In some patients the intensity of the cytoplasmic ERβ staining of the OT neurons was stronger than that in AVP neurons (Table 1). It did not show clear sex differences, but decreased during the course of aging. In addition, OT cells in the SON, when stained with anti-ERα antibody, showed moderate cytoplasmic staining regardless of age and sex.

Pathological parameters

Fixation time was not correlated to the percentage of 1) nuclei staining for ERβ ($P = 0.305$) or ERα ($P = 0.608$), 2) cells with positive cytoplasm for ERβ ($P = 0.235$) or ERα ($P = 0.717$), and 3) ERβ ($P = 0.235$)- or ERα ($P = 0.717$)-negative cells. Postmortem delay appeared to be significantly correlated to the number of cells showing ERβ ($P = 0.017$) and ERα ($P = 0.012$) nuclear staining. This correlation could, however, be fully explained by the accumulation of subjects with long postmortem times in the younger age group. As there was no difference in postmortem delay between young males and young females ($P = 0.876$), whereas only young women showed a prominent nuclear ERβ staining, this difference cannot have influenced the sex differences in our data.

Discussion

The results of the present study demonstrate the presence of ERβ and ERα in AVP neurons of the human SON and their differential expression in relation to age and sex. As nuclear staining of ERs is considered to be bound and stimulating, cytoplasmic to be unbound and not active (see below), it is of great interest that nuclear and cytoplasm ERβ-positive AVP neurons were found predominantly in young women, whereas in young men and elderly women more nuclear ERα-positive AVP cells were observed. In our previous studies (6, 7) we showed that AVP neurons in young women are less active than those in young men or elderly women. Our current data show that ERβ immunoreactivity is stronger, and the proportion of ERβ-positive AVP neurons is highest in young women and lowest in young men. Elderly women showed decreased expression of ERβ in the SON. This indicates that the inhibitory role of estrogens in AVP neuron activity is probably mediated via the increase in ERβ and the

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TABLE 3. The mean percentage of ERα-stained AVP neurons in the SON in different age groups

	Age ≤50 yr		Age >50 yr	
Sex	Male	Female	Male	Female
No.	8	6	8	10
Nucl+ (%)	6.35 ± 3.1[a,b]	1.9 ± 0.9[a]	3.3 ± 0.9[b]	9.03 ± 2.4
Cytopl+ (%)	96.6 ± 1.9	88.5 ± 6.2	97.8 ± 1.4	96.9 ± 2.6
Neg− (%)	3.4 ± 1.9	11.5 ± 6.2	2.2 ± 1.4	3.1 ± 2.6

Nucl+ (%), The percentage of cells with the nuclear staining; Cytopl+ (%), the percentage of cells with the cytoplasmic staining; Neg− (%), the percentage of negative cells.

[a] Statistically significant difference between young men and young women.

[b] Statistically significant difference between young and elderly men.

decrease in ERα (6, 7). Activation of female AVP cells in aging, as shown previously (6, 7), may thus occur as a result of the drop in estrogen levels after the menopause (19), a subsequent loss of ERβ in AVP neurons, and an increase in ERα, resulting in diminished inhibition on these neurons. Indeed, it was previously shown that ovariectomy in the rat caused an increase in plasma AVP levels (27) and in the neurosecretory activity of SON neurons (28) in females. One case in our study (no. 80002), a 46-yr-old woman who underwent a bilateral ovariectomy 22 months before her death and, hence, had low estrogen levels, deserves special attention. No nuclear staining of ERβ was found in that patient, whereas prominent cytoplasmic ERβ staining was marked in both AVP and OT neurons. Interestingly, when stained for ERα, this patient showed the strongest nuclear staining. This observation supports the idea of ERβ-mediated inhibition and ERα-mediated stimulation of AVP cells by estrogens acting at the genomic level. The observed sex differences in ERβ expression support the previous reports (6, 7) that the activity of AVP neurons in young women may be suppressed directly by estrogens via ERβ when small amounts of ERα are present. It was demonstrated *in vitro* in a GH_3 cell line that estrogens up-regulate ERβ expression (25) and down-regulate ERα in some rat brain areas (29), indicating that the effects of estrogen on ER expression are region specific (29). Moreover, it has been shown recently that in the same region (in rat dorsal root ganglion neurons) long-term estrogen treatment of ovariectomized rats down-regulates the levels of ERα mRNA and up-regulates the levels of ERβ mRNA (30), which is in line with our data about the differential expression of ER subtypes in relation to age and sex. In the rat an alternative *trans*-synaptic regulation of SON neurons by estrogens from lamina terminalis and preoptic area projecting to the SON has, in addition, been proposed (31). Whether a similar mechanism of SON regulation also operates in the human hypothalamus is not known at present.

In contrast with ERβ, a totally different pattern of ERα staining was observed in the AVP cell population. Men and elderly women showed more nuclear and cytoplasmic positive neurons than young women. More negative ERα cells were observed in young women than in any other group studied. Our data are fully in agreement with the proposed antagonistic roles of ERβ and ERα in HeLa cells *in vitro*, where ERα-activated and ERβ-inhibited transcription (15). Thus, in young women in whom ERβ immunoreactivity is high, ERα expression is significantly lower, while in elderly women and young men, in whom ERβ immunoreactivity is negligible, ERα is abundantly expressed. A large body of evidence in animal experiments suggested differential roles of ERβ and ERα. Thus, in the rat, mRNAs and peptides of these two ER subtypes appeared to be differentially localized not only in the hypothalamus (32, 33), but also in the ovary and uterus (34–36). Moreover, the content of ERβ in female rhesus macaques was higher than that in males (37). It was further suggested that ERβ and ERα may differ in transcriptional activities (38).

Nuclear/cytoplasmic ER staining

In the SON of rat (8, 9) and monkey (11), ER immunoreactivity was described in both the nucleus and the cytoplasm. The presence of nuclear ERs in neurosecretory cells in animals (8, 9, 11, 17, 18) and humans, as appears from the present study, suggests a direct genomic regulatory effect of estrogens in AVP neurons. It has been well demonstrated that both liganded and unliganded ERs are localized in the nucleus of the neurons (39) and that unliganded ERs are present in both the nucleus and the cytoplasm of neurons, including dendrites and axonal terminals (40–42). Cytoplasmic immunostaining was eliminated 1 h after 17β-estradiol administration, probably due to conformational changes in the receptor (43). This means that if estrogens strongly affect cell function they are mainly present in the nucleus and to a much lesser degree in the cytoplasm. Steroid receptors continuously shuttle between the nucleus and cytoplasm by both diffusion and active transport (44). In addition, it was shown in the rat that high levels of ERs coincide with the preovulatory estrogen level surge (45), suggesting ER (probably ERα) up-regulation in the brain tissue by estrogens. All observations to date indicate that binding of the appropriate hormonal ligand to the receptor activates the receptor by phosphorylation, resulting in its movement from the cytoplasm to the nucleus (44). This sequence of events fully agrees with our observation of a high proportion of SON neuronal nuclei staining for ERβ exclusively in young females. The existence of a sex difference may, in principle, be due either to organizational effects during development or to activational effects of sex steroids in adulthood (46). Our data show an inhibitory effect of estrogens on AVP neurons depending on circulating levels of estrogens in adulthood and possibly mediated by ERβ (Refs. 6 and 7 and the present study). The reported sex difference in ERβ and α should thus be interpreted as "activational inhibitory" effects in adulthood.

Perinuclear and nucleolar staining

In 8–54% of the SON neurons we noticed a clear ERβ perinuclear staining. This staining followed the pattern observed in cytoplasmic staining concerning its sex and age differences. According to the observations of Eneström (26), in the rat this band might be the perinuclear part of the granular endoplasmic reticulum (nuclear envelope), which is consistently proliferating, and its outer leaf invaginates into the perikaryon. Indeed, we observed a similar perinuclear band in thionine-stained sections in the SON of the patients

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studied, suggesting that the nucleus of the AVP neurons in the human dl-SON is also surrounded by the perinuclear part of the endoplasmic reticulum. Fewer (1–9%) ERα-stained neurons showed perinuclear staining.

Interestingly, we observed nucleolar staining in ERα-stained cells in several cases, predominantly in young men and elderly women. This localization is in agreement with the study in human breast cancer epithelial cell lines, where nucleolar staining was also found with ERα and was suggested to be a consequence of the mechanism involved in ER down-regulation (47), and is thus consistent with our data showing lower ERα expression in the SON in young women compared to other groups.

ERβ and ERα in OT neurons

In OT neurons, very prominent ERβ cytoplasmic staining without nuclear staining was generally present. Only in two cases was nuclear staining found for ERβ. We cannot speculate on the reason for the weak nuclear staining in patient 92047, but in the case of patient 94040 reanimation was performed with high doses of vasoactive drugs followed by a cardiogenic shock that may have influenced these SON cells. In a few cases OT neurons in the SON stained more intensively for ERβ than AVP cells. We did not find a clear sex difference in ERβ immunoreactivity in OT neurons, whereas a gradual decrease in ERβ cytoplasmic staining was observed in aging. ERα expression in the SON OT cells showed only moderate cytoplasmic staining regardless of age or sex. This was unexpected, because in animals OT neurons were found to express both ERβ and ERα immunoreactivities (17, 18, 48), and the OT gene contains estrogen-responsive elements in the rat (49) and human (50). The large body of experimental evidence suggests that estrogens up-regulate OT production in the rat (51–54). It was, however, further suggested that estrogens not only directly regulate genes present in OT neurons via estrogen receptors (9, 17), but also exert their action at the OT cell membrane level (55), which may explain the absence of sex differences in ER staining in the OT neurons of the SON. It should also be noted that OT neurons of the SON represent only a small number per case, and it is well known that the majority of OT neurons are located in the paraventricular nucleus (1), which is the subject of future study.

In summary, our results demonstrate for the first time differential expression of ERβ and ERα in the human SON that is strongly influenced by age and sex in an antagonistic way. The decreased ERβ and increased ERα staining in postmenopausal women are probably essential parts of the mechanism of activation of AVP neurons in this group of subjects. The activation of AVP neurons in postmenopausal women may be at least a part of the explanation for the frequent occurrence of hypertension and other cardiovascular diseases in this group of people.

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