

Expression of Estrogen Receptor- β Messenger Ribonucleic Acid in Oxytocin and Vasopressin Neurons of the Rat Supraoptic and Paraventricular Nuclei*

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ABSTRACT

The regulatory actions of estrogen on magnocellular oxytocin (OT) and vasopressin (VP) neurons of the paraventricular (PVN) and supraoptic (SON) nuclei are well documented. To date it is still debated whether the effect of estrogens is exerted directly or mediated by estrogen-sensitive interneurons. Previous immunocytochemical (ICC) and *in situ* hybridization (ISH) studies detected either low levels or absence of the classical estrogen receptor (ER- α) in the PVN and the SON of the rat. The present experiments using a combined ICC and ISH method were undertaken to examine the expression of the recently cloned beta form of ER (ER- β) in OT- and VP-immunoreactive (IR) neuronal systems of the rat hypothalamus. The results demonstrate that the highest cellular levels of ER- β messenger RNA (mRNA) in OT-IR neurons can be visualized in the caudal portion of

the PVN and in an area ventro-medial to the central core of VP-IR cells. These neurons were previously shown to project caudally to the brain stem and the spinal cord to regulate autonomic functions. In addition, the whole rostro-caudal extent of the PVN and the SON contained OT-IR neurons that coexpressed variable levels of ER- β mRNA. Similarly, the presence of ER- β mRNA was seen in a large population of VP-IR paraventricular and supraoptic neurons. In the SON, somewhat stronger hybridization signal was detected in VP-IR neurons as compared with OT-IR neurons.

Together, these findings provide strong support for the concept that the functions of OT- and VP-IR neurons in the PVN and the SON are regulated directly by estrogen and that the genomic effects of estrogens are mediated by ER- β . (*Endocrinology* **139**: 2600–2604, 1998)

THE MAGNOCELLULAR neurosecretory system consists of vasopressin (VP)- and oxytocin (OT)-synthesizing neurons that send axon projections to the neurohypophysis from the hypothalamic supraoptic and paraventricular nuclei (SON; PVN) (1). Whereas the main function attributed to VP is the central control of water and electrolyte balance (2), OT is primarily involved in the regulation of reproductive functions, including parturition (3), sexual (4, 5), and maternal (6) behavior, and the ejection of milk (7). The neurohormone output from OT-ergic and VP-ergic neurons is controlled partly by neuronal mechanisms (7). Among the humoral agents influencing OT and VP neurons, the actions of ovarian steroids have been studied extensively. The observations that the expression of OT messenger RNA (mRNA) in magnocellular neurons increases with the onset of puberty and decreases following castration (8), and that the cellular levels of OT mRNA exhibit variations during the estrus cycle (9) indicate that estrogen regulates the expression of OT. Furthermore, increased VP, in addition to OT mRNA levels, was detected in the SON of pregnant and lactating rats (9). While the effects of estrogen on magnocellular functions are well documented, the site of

estrogen action, direct or interneuron-mediated, remains a question of controversy. Although ER immunoreactivity was colocalized with the majority of OT neurons in the guinea pig (10), ligand binding (11–13), ICC (14), and ISH (15) studies failed to demonstrate estrogen-receptivity of most magnocellular neurons in the rat. The observations of Herbison *et al.* (14) indicate that ER-IR GABA-ergic neurons located in the perinuclear zone of the SON might mediate, at least in part, the effect of estrogen on OT neurons in this species.

Recently, a new estrogen receptor, termed ER- β , was cloned from the rat prostate gland (16). *In situ* hybridization studies with ER- β -specific complementary RNA (cRNA) probes have established a wide distribution of ER- β mRNA in the hypothalamus (17) and brain (18) of the rat. One interesting finding of these experiments was the high level of ER- β mRNA in the PVN and the SON. The aim of the present studies was to analyze the expression of ER- β mRNA in OT- and VP-IR neurons of the hypothalamic PVN and SON, by means of a combined ICC and ISH method (19).

Materials and Methods

Preparation of hybridization probes

The preparation of hybridization probes was described earlier (17). Briefly, a 285- and 558-bp fragment (bases 52–610 and 1809–2094, respectively) of the rat ER- β cDNA (16) were selected as templates for transcription of cRNA probes and subcloned into the *Eco*RI site of the pBluescript plasmid vector (Stratagene, La Jolla, CA). The vectors were linearized with *Bam*HI and antisense RNA probes transcribed using the T7 promoter in the presence of 35 S-UTP. To increase detection sensitivity

Received November 7, 1997.

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* This work was supported, in part, by grants from the National Science Foundation of Hungary (Nos. TO-16354 and F-22711).

of the hybridization procedure, the two probes were preferentially combined in a hybridization cocktail, instead of using a single probe. A series of preliminary experiments detailed elsewhere verified the specificity of these hybridization probes (17).

ISH detection of ER- β mRNA by autoradiography

For single-labeling ISH studies of ER- β mRNA expression, fresh-frozen sections including the hypothalamic PVN and SON of the ovariectomized female rat were used as described earlier (18). For single-cell analysis of the hybridization signal, the autoradiograms were visualized on Kodak NTB-3 photographic emulsion.

Combined use of ICC and ISH in the same sections

The methodology of combined ICC and ISH was modified from a previous protocol (19).

Animals

Five female Wistar rats (200–220 g) were maintained in a controlled environment (5-h light, 19-h dark, 22 C), with food and water available *ad libitum*. The animals were anesthetized with Avertin (1 ml/100 g BW), and gonadectomized bilaterally. On postovariectomy day 12, the animals were anesthetized and killed by transcardiac perfusion using a short flush with 0.1 M PBS (pH 7.6), followed by 400 ml of a 4% paraformaldehyde solution in PBS. Small tissue blocks containing the hypothalamic SON and PVN were dissected from the brains, infiltrated with gradually increasing concentrations of sucrose through 30% (for 24 h total), and snap-frozen on dry-ice. Then, 20 μ m coronal sections were prepared on a freezing microtome (Reichert-Jung), collected and stored in a cryoprotectant solution at -20 C (20) until use.

Preincubation, hybridization, and washes

Immediately before hybridization, the sections were removed from the cryoprotectant solution and rinsed thoroughly in 2 \times standard saline citrate solution (2 \times SSC; 1 \times SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). Subsequently, they were pretreated with proteinase K (Serva Feinbiochemica GmbH & Co., Heidelberg, Germany; 1 μ g/ml, dissolved in 2 \times SSC at room temperature) for 30 min, postfixed with 4% paraformaldehyde for 5 min to stop the digestion, rinsed shortly in 2 \times SSC, and acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0)/0.9% NaCl for 20 min. Finally, the sections were rinsed in 2 \times SSC and transferred into hybridization solution.

During the hybridization, the sections were incubated in microcentrifuge tubes containing the hybridization cocktail [50% formamide, 4 \times SSC, 10% dextran sulfate (500,000 mol wt; Sigma Chemical Co., St. Louis, MO), 1 \times Denhardt's solution, 500 μ g/ml heparin sodium salt (Sigma), 0.5 mg/ml yeast tRNA (Boehringer Mannheim GmbH, Mannheim, Germany), 0.4 mg/ml sheared single-stranded salmon sperm DNA (Boehringer Mannheim GmbH), 200 mM dithiothreitol, and 30,000 cpm/ μ l of each hybridization probe. The tubes were sealed with Parafilm to exclude atmospheric air and placed in an incubator for hybridization overnight at 52 C.

In the morning, the hybridized sections were rinsed in 1 \times SSC for 10 min, followed by a 1-h stringency wash in 50% formamide/2 \times SSC at 50 C and a 1-h digestion of excess hybridization probes with RNase A (Boehringer Mannheim GmbH; 100 μ g/ml in 2 \times SSC buffer) at 37 C. Finally, two sequential stringent treatment steps (1 h each) were performed in 50% formamide/2 \times SSC solutions at 50 C and the sections were rinsed in 0.1 M PBS with 0.2% Triton X-100.

Immunocytochemical detection of oxytocin and vasopressin

Before the immunocytochemical detection of either OT or VP was carried out, the sections were immersed in 0.5% H₂O₂ in PBS for 10 min and blocked with 2% BSA against nonspecific antibody-binding for 20 min. The primary monoclonal antibodies for neurophysin-OT (PS-38; 1/1000, kindly provided by Dr. Sharon Key, Public Health Service, NIH, Bethesda, MD) were diluted with 2% BSA in PBS and applied to the sections for 12 h at 4 C. The tissue-bound antibodies were reacted with biotinylated antimouse IgG (1/800; Jackson ImmunoResearch Labora-

tories, Inc., West Grove, PA), then with streptavidin-peroxidase complex (1/2000; Jackson ImmunoResearch Laboratories, Inc.) for 1 h each. The antiserum against VP (Incstar Corp., Stillwater, MN) was raised in a rabbit and applied to the sections at a 1/4000 working dilution for 12 h. The detection protocol included sequential incubation of sections in the antirabbit IgG (1/500, Arnel Products Co., Inc., New York, NY) and then, in the peroxidase-antiperoxidase complex (1/2000, Dako Corporation, Carpinteria, CA) solutions for 1 h each. Finally, OT- and VP-IR neurons were visualized in a developer containing 0.01% diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich Chemical Company) and 0.003% H₂O₂ in 0.1 M Tris-HCl buffer (pH 7.6).

Detection of the hybridization signal

The immunostained sections were rinsed in PBS for 5 min, transferred briefly into 0.3% Elvanol with 300 mM ammonium acetate, mounted onto precleaned double gelatin-coated microscopic slides, and air-dried. They were then dehydrated in a graded series of ethanol through 100%, dried, and then dipped into photographic emulsion (LM-1; Amersham International plc, Amersham, UK) and exposed for 3–6 weeks at 4 C. The slide autoradiograms were developed with Kodak D19 developer for 3 min, rinsed briefly with distilled water, then fixed with Kodak fixer for 5 min. Finally, the sections were dehydrated in ethanols, cleared in xylenes and coverslipped with Permount.

Mapping of double-labeled cells

The immunocytochemical distribution of OT- and VP-IR neurons did not seem to respect the borders of the classical anatomical subnuclei of the PVN. Moreover, a high percent of OT-IR neurons occurred in the classical parvocellular subnuclei of the PVN (21). Therefore, the topographical analysis of double- and single-labeled neurons in the PVN followed a simplified terminology adapted from a previous immunocytochemical study of OT- and VP-IR neurons, which subdivided the PVN into a rostral (rPVN) a middle (mPVN) and a caudal (cPVN) portion (22).

In the caudal portion of the PVN, where the highest cellular levels of ER- β mRNA were detected, a semiquantitative estimate of the numbers of ER- β -positive OT-IR neurons was made. From sixteen sections representing the cPVN of four animals, a total of 543 OT-IR neurons were analyzed for co-expression of ER- β mRNA.

Results

Single-labeling studies

Following 3–6 weeks of autoradiographic exposure, a clear hybridization signal was detectable in all three rostro-caudal subdivisions of the PVN and the whole rostro-caudal extent of the SON. In order, the strongest labeling of neurons was observed in the cPVN (Fig. 1a), then in the mPVN (Fig. 1b) and finally, in the rPVN (not shown) and the SON (Fig. 1c). In the SON, the signal had a tendency to be slightly higher in the ventral than in the dorsal part of the nucleus.

Colocalization studies with combined use of ICC and ISH

The method of combined ICC and ISH was used for the simultaneous visualization of OT- or VP-immunoreactivity and ER- β mRNA (Fig. 2).

Paraventricular nucleus

Corroborating the results of single-labeling experiments, the peak ISH signal intensity was observed in the cPVN. The majority of the high ER- β mRNA-expressing neurons in the cPVN were immunoreactive for OT (Fig. 2, a and b). Semiquantitative analysis of OT neurons further revealed that 93 \pm 6.8% also expressed ER- β hybridization signal. In addition, the few VP-IR neurons that intermingled with OT

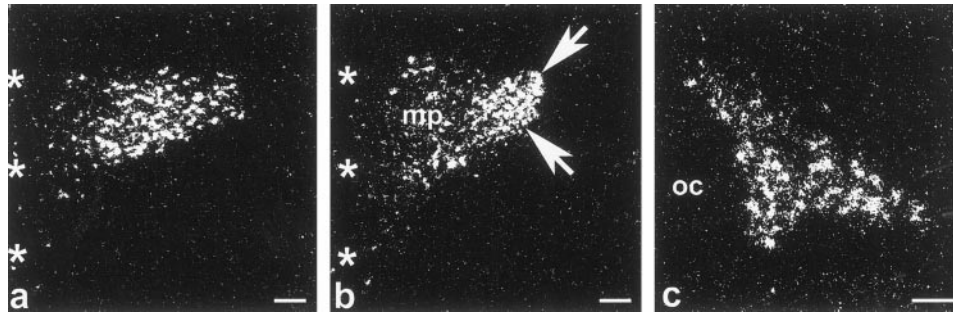


FIG. 1. Detection of ER- β mRNA in representative sections of paraventricular (PVN) and supraoptic (SON) nuclei by ISH. The expression of ER- β mRNA in the PVN is highest in the caudal part (a) and attenuated in the middle part of the nucleus (b). The receptor signal is the strongest in the lateral, magnocellular subdivision (*arrows*), whereas the medial, parvocellular subdivision (mp) is weakly labeled. In the SON (c) the hybridization signal is slightly higher in the ventral than in the dorsal part of the nucleus. *, Third ventricle; oc, optic chiasm. Scale bars, 150 μ m (in a, b) and 100 μ m (in c)

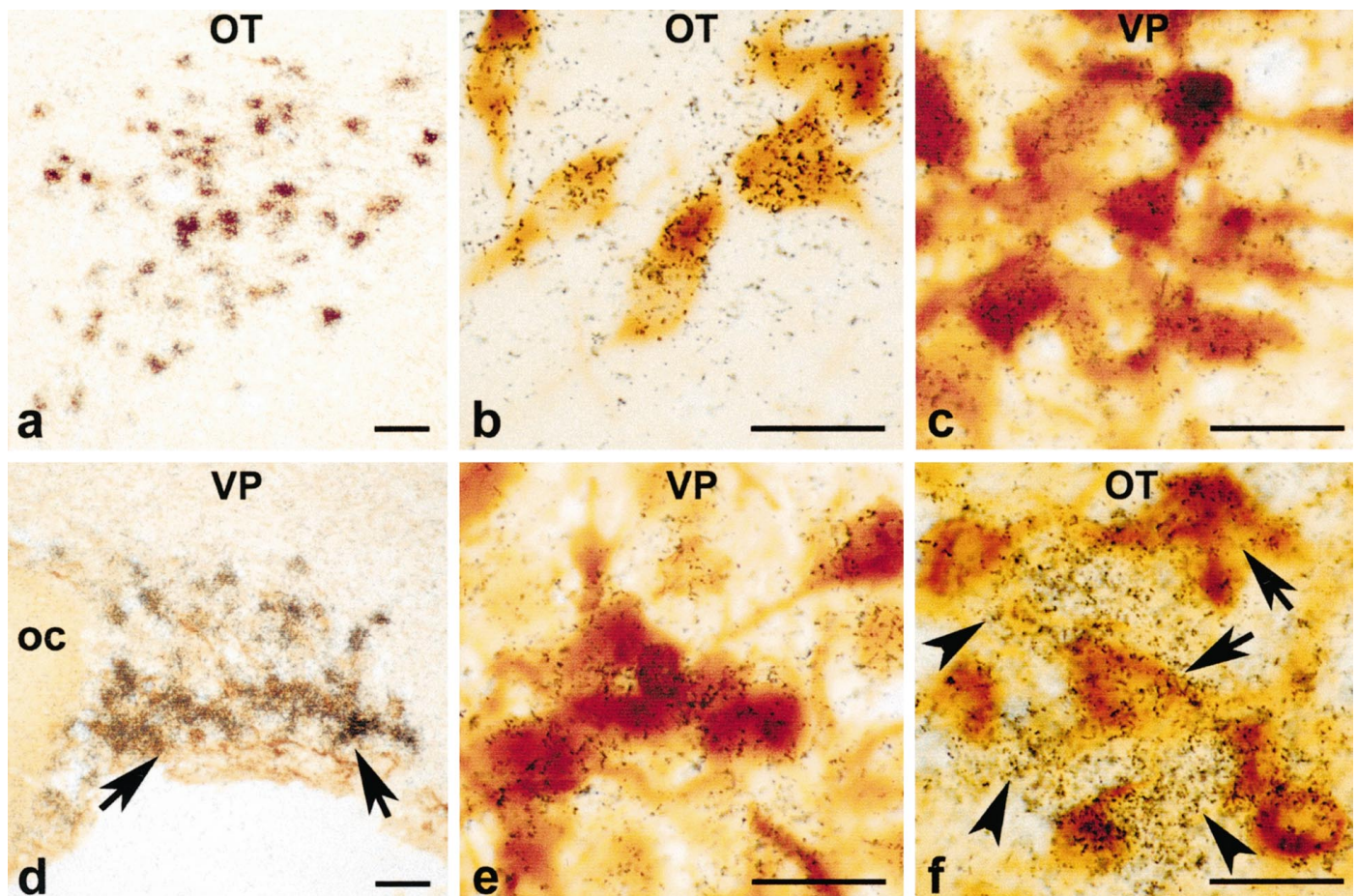


FIG. 2. Expression of mRNA encoding the β type of ER in OT- and VP-immunoreactive (IR) perikarya located in the hypothalamic paraventricular (PVN) (a–c) and supraoptic (SON) (d–f) nuclei of OVX rats. oc, Optic chiasm; OT, oxytocin; VP, vasopressin. a, Most of the OT-IR neurons in the caudal part of PVN exhibit strong hybridization signal for ER- β mRNA. In this part of the nucleus, the OT-IR neurons are scattered rather than forming a solid mass. b, OT-IR/ER- β mRNA, and c, VP-IR/ER- β mRNA double-labeled cells at higher magnification in the PVN. d, ER- β mRNA expression in VP-IR neurons of SON. The autoradiographic silver grains are mainly clustered over VP-IR neurons located in the ventral part of the nucleus (*arrows*). e, VP-IR/ER- β mRNA, and f, OT-IR/ER- β mRNA double-labeled cells shown at a higher power in the SON. In f, *arrows* point to double-labeled cells, *arrowheads* mark immunonegative cells that express the ER- β mRNA. Scale bars, 60 μ m (in a, d) and 25 μ m (in b, c, e, f).

neurons in the cPVN often coexpressed detectable, albeit relatively lower levels of ER- β mRNA. In the mPVN, the strongest hybridization signal was detected in cells situated ventro-medial to the central core of VP neurons, and dual-

labeling studies with the OT antiserum have established that most of them were OT-ergic. Variable levels of ER- β mRNA were revealed in different populations of OT- as well as VP-IR (Fig. 2c) neurons. Subsets of OT- and VP-IR neurons

in the mPVN did not contain detectable hybridization signal. Similarly, the population of OT-IR neurons in the rPVN consisted of a mixture of ER- β mRNA-expressing and ER- β mRNA-negative neurons, reflecting either lack of ER- β -coexpression in some OT-ergic neurons or a limitation of the method.

Supraoptic nucleus

In the SON, ER- β mRNA was coexpressed both in VP- (Fig. 2, d and e) and OT-IR (Fig. 2f) neurons. Somewhat higher cellular levels of the receptor mRNA were seen in VP as compared with OT neurons. Dual-labeled cells appeared along the rostro-caudal extent of the nucleus. A longer exposure time was needed to visualize the hybridization signal in the SON, compared with the PVN.

Discussion

The results of the studies described herein have demonstrated that ER- β mRNA is expressed in OT- and VP-IR neurons of the hypothalamic PVN and SON.

Substantial data support an important role for estrogen in the regulation of magnocellular OT and VP neurons. The concentration of OT in the pituitary (9) and the hypophyseal portal blood (23) as well as OT mRNA levels in the SON (9) change throughout the estrus cycle and estrogen treatment of ovariectomized rats increases plasma OT levels (24). In addition, VP mRNA in the SON is elevated in lactating and pregnant rats (9), and lactation also increases the percent ratio of OT neurons that coexpress VP mRNA in this nucleus (25). The effects of estrogen on the magnocellular systems are well documented, although whether they are direct or interneuron-mediated have long been a question of controversy. In the guinea pig, most OT neurons of the PVN and the SON contain immunoreactive ER (10), supporting the hypothesis of a direct effect of estrogen. In contrast, few magnocellular neurons were found to contain ER immunoreactivity in sheep (26) and monkeys (27). In rats, only a restricted population of paraventricular neurons concentrate tritiated estradiol (11–13). *In situ* hybridization histochemical studies of the rat hypothalamus have revealed the absence of ER- α mRNA in the SON and PVN (18). Therefore, the concept that the actions of estrogen on OT and VP neurons are mediated by estrogen-sensitive interneurons has been raised. Accordingly, an ER-IR GABA interneuron population located in the perinuclear zone of the SON has been implicated in the circuitry, whereby estrogens might regulate OT neurons of the SON (14).

Recently, a second form of ER, termed ER- β , has been cloned from rat prostate (16). Using ISH with complementary probes to the ER- β mRNA, the anatomical distribution of ER- β mRNA-expressing cells has been mapped in the hypothalamus (17) and brain (18) of the rat. A surprising observation of these studies was the wide distribution of ER- β mRNA in the PVN and the SON. We now show that a large percent of OT- and VP-IR neurons in these nuclei coexpresses ER- β mRNA. The highest hybridization signal was revealed in the cPVN, where $93 \pm 6.8\%$ of OT-IR neurons expressed detectable levels of ER- β mRNA. Interestingly, this topographical distribution of double-labeled OT neurons highly

resembles the map of neurophysin-containing neurons that exhibit nuclear uptake of tritiated estradiol (12, 13), and project caudally to the medulla and/or spinal cord to influence autonomic functions (21). Nevertheless, the area populated by ER- β mRNA-positive OT neurons is larger and includes the whole rostro-caudal extent of the PVN as well as the SON, comprising regions that project to the posterior lobe of the pituitary gland. Most VP-ergic neurons expressing ER- β mRNA were localized in the middle portion of the PVN (mPVN) and ventrally in the SON. To establish the precise role of ER- β in functionally distinct subpopulations of OT and VP neurons and to identify parvocellular neuropeptide systems that express ER- β mRNA in the subnuclei of the PVN, further studies will be required. The genes regulated by ER- β in OT and VP neurons are presently unclear. However, identification of ER- β mRNA in OT neurons, most of which do not express ER- α , might explain the presence of functional estrogen-response elements on the promoter of the OT gene, itself (28). Among the neuropeptides that have been colocalized in magnocellular VP neurons (29), galanin might represent a putative target for regulation by ER- β because of the presence of estrogen-response elements on the preprogalanin gene (30).

The method of combined ICC and ISH has been used successfully earlier for the colocalization of preprogalanin mRNA with c-Fos- and LHRH immunoreactivities in the preoptic area of the female rat (19). In the present application of the combination technique, several efforts have been made to maximize the sensitivity of the ISH detection component. This seemed to be especially important in view of the relatively low cellular abundance of ER- β mRNA. Modifications we have introduced to the protocol included a proteinase K digestion step before hybridization to facilitate access of probes to the target mRNA and combination of two antisense hybridization probes in the hybridization cocktail, instead of using a single cRNA probe. These changes markedly increased the sensitivity of the hybridization.

In summary, the present studies have localized ER- β mRNA in paraventricular and supraoptic OT-IR and VP-IR neurons of the rat. These new data suggest that estrogen directly regulates gene(s) in OT-IR and VP-IR neurons.

Acknowledgments

The authors wish to express their sincere gratitude to Ms. Mónica Kara, Ms. Andrea Kobolák, and Mr. Malcolm Lane for the excellent technical assistance. Dr. Sharon Key is thanked for the kind donation of the oxytocin antibody.

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