Regulation of Estrogen Receptor Messenger Ribonucleic Acid in Rat Hypothalamus by Sex Steroid Hormones

Richard B. Simerly and Beckie J. Young

Howard Hughes Medical Institute
The Salk Institute for Biological Studies
La Jolla, California 92037

Sex steroid hormone receptors are thought to mediate the actions of their respective hormones by functioning as ligand-activated nuclear transcription factors that alter the expression of specific sets of hormone-responsive genes. Particularly high densities of estrogen receptor (ER)-containing neurons are located in the arcuate nucleus (ARH) and ventrolateral part of the ventromedial nucleus (VMHvl) of the hypothalamus, and these cell groups are thought to play key roles in the neuroendocrine control of reproductive function. Thus, hormonal regulation of ER gene expression in ARH and VMHvl neurons represents a direct mechanism by which circulating sex steroids could affect the responsiveness of these neurons to hormonal activation. We used *in situ* hybridization histochemistry to evaluate the influence of estradiol and testosterone on levels of ER mRNA within the ARH and VMHvl of adult male and female rats. In female rats, estradiol treatment reduced levels of ER mRNA in the ARH and VMHvl within 24 h relative to levels in both ovariectomized control animals and intact estrous females. Comparable results were obtained in male rats, except that testosterone did not significantly attenuate ER mRNA hybridization in the VMHvl until after 3 days of hormone treatment, and only a minor decrease was noted in the ARH, which was not statistically significant. In both male and female animals, the overall density of labeling found over individual cells in emulsion-dipped autoradiograms was consistently lower in hormone-treated animals compared with that over cells in gonadectomized controls, suggesting that the observed decreases in ER mRNA hybridization measured over the ARH and VMHvl are due to changes in cellular levels of ER mRNA. These results indicate that circulating sex steroids suppress the biosynthesis of the ER in ARH and VMHvl neurons, thereby altering the responsiveness of these cells to hormonal activation. (Molecular Endocrinology 5: 424–432, 1991)

0888-8809/91/0424-0432$03.00/0
Molecular Endocrinology

INTRODUCTION

Circulating sex steroid hormones influence the activity and development of hormone-sensitive neural circuitry in the brain through interactions with specific saturable receptors that are found in discrete populations of neurons. Cells that express estrogen receptors are particularly abundant in the arcuate (ARH) and ventromedial (VMH) nuclei of the rat hypothalamus (1–8), and these nuclei are known to be critical components of the neural circuitry controlling gonadotropin secretion and copulatory behavior (9, 10). The cellular basis for much of estrogen's regulatory influence on reproductive function is generally thought to involve a ligand-mediated activation of the receptor protein, which, in turn, acts at the genomic level to alter the expression of specific sets of genes that regulate neuronal function (11–15). Thus, the estrogen receptor (ER) appears to function as a ligand-activated transcription factor that regulates the expression of genes encoding neurotransmitter molecules (16–23), certain rate-limiting biosynthetic enzymes (24–27), or other regulatory proteins (28–32), which mediate many of the effects of estrogens on brain function.

Estrogen treatment has been shown to decrease levels of ER immunoreactivity in both rat (Ref. 1; but see also Ref. 6) and guinea pig brain (33–35), indicating that cellular levels of the ER itself may be sensitive to changes in levels of circulating sex steroids. In addition, treatment of gonadectomized male and female rats with sex steroids increases levels of estrogen binding in nuclear extracts (36–39), although these findings are generally thought to reflect translocation of the activated hormone-receptor complexes to the nuclear compartment (14) or hormone-induced changes in the affinity of the receptor for chromatin and other nuclear components (12, 15).

That sex steroids exert regulatory effects on the biosynthesis of the ER has been addressed in a variety of tissues and cell lines. Estradiol (E_2) decreases ER protein and mRNA levels in MCF-7 cells (40–43), but was found to either increase (42) or not affect (40) ER expression in T47D breast cancer cells. Furthermore,

androgens were also found to suppress ER protein and mRNA levels in ZR-75–1 breast cancer cells (44). Although E_2 suppresses levels of ER mRNA in rat uterus, it has the opposite effect in the liver and pituitary (45). Thus, circulating sex steroids appear to exert both tissue- and cell-specific regulatory influences on ER biosynthesis.

The regulatory profile of ER gene expression in the brain has not been established. Both the low abundance of ER mRNA within individual neurons and the relatively few cells that express the ER in any particular brain region make it difficult to detect hormonally induced changes in ER mRNA within discrete populations of neurons by using conventional biochemical methods. In the present study, *in situ* hybridization was used to evaluate the influence of circulating sex steroids on ER mRNA within cells of the arcuate nucleus (ARH) and ventromedial nucleus (VMH) of the hypothalamus in male and female rats. The results indicate that sex steroids down-regulate ER gene expression in ARH and VMH neurons and thereby influence the sensitivity of cells in these two important neuroendocrine nuclei to hormonal activation.

RESULTS

Heavily labeled ER mRNA-containing neurons were found in the ARH, with very few labeled cells in the cell-poor zone lateral to the nucleus (Fig. 1) (see Ref. 46 for review of hypothalamic cytoarchitecture). The ventrolateral part of the ARH (VMHvl), which in Nissl-stained material is characterized by its medium-sized darkly stained cells, contained the greatest density of heavily labeled ER mRNA-containing cells. At middle levels through the ARH the distribution of these heavily labeled cells extended ventrolaterally beyond the morphological borders of the nucleus toward the base of the brain. The small-celled dorsomedial part of the ARH contained fewer ER mRNA-containing neurons that had only low to moderate densities of overlying silver grains. Nearly all of the ER mRNA-containing cells found in the VMH were localized to its ventrolateral subdivision (VMHvl), and these cells were very heavily labeled. In addition to the ER mRNA-containing neurons in the VMHvl, several moderately labeled cells were found in the adjacent part of the lateral hypothalamic area, located lateral and dorsal to the VMH, but these cells were not included in the quantitative analysis described below.

Hormonal Regulation of ER mRNA

The possible regulation of ER mRNA levels in cells of the ARH and VMHvl by circulating gonadal steroids was evaluated in male and female rats. Two separate experiments were carried out to, first, evaluate the influence of E_2 treatment on ER mRNA hybridization in the ARH and VMHvl of female rats and, second, evaluate the influence of circulating testosterone (T) on ER mRNA hybridization in the same cell groups of male rats.

Females

Treatment of ovariectomized female rats with E_2 appeared to down-regulate levels of ER mRNA in the ARH and VMHvl at all time intervals studied. ER mRNA hybridization was decreased in the ARH by approximately 40% in ovariectomized animals treated with E_2 for 24 h (Fig. 1) relative to that in ovariectomized animals that received control pellets (CP) for 24 h and that in intact estrous females (Fig. 2). ER mRNA hybridization was also decreased in animals treated for 3 or 7 days compared with either levels in intact or ovariectomized control animals treated and processed in parallel, suggesting that this suppression is maintained for at least 7 days. No significant differences in ER mRNA hybridization were found between the intact estrous females, which had low plasma levels of E_2 (~47 pg/ml), and the CP-treated ovariectomized animals, in which plasma E_2 was undetectable. Overall levels of ER mRNA hybridization appeared to be substantially greater in the VMHvl, because signals were obtained over the VMHvl with 4-day exposures that were comparable to those

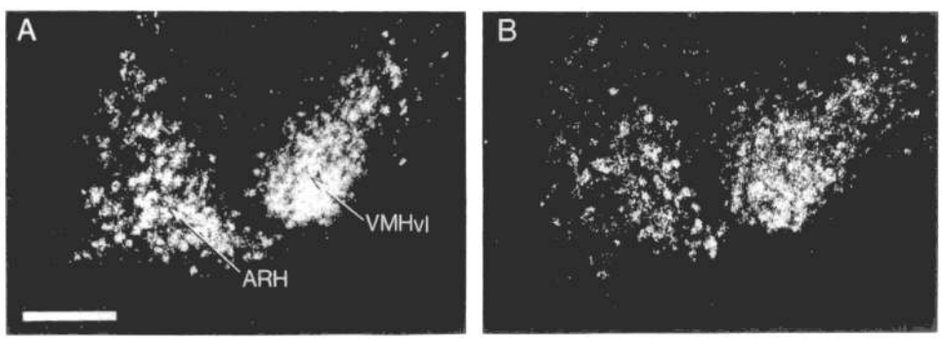

Fig. 1. ER mRNA in the ARH and VMHvl

Darkfield photomicrographs that compare the distribution and intensity of labeling over the ARH and VMHvl in ovariectomized female rats after 1 day of treatment with a control pellet (A) or E_2 (B). *Scale bar* = 160 μm.

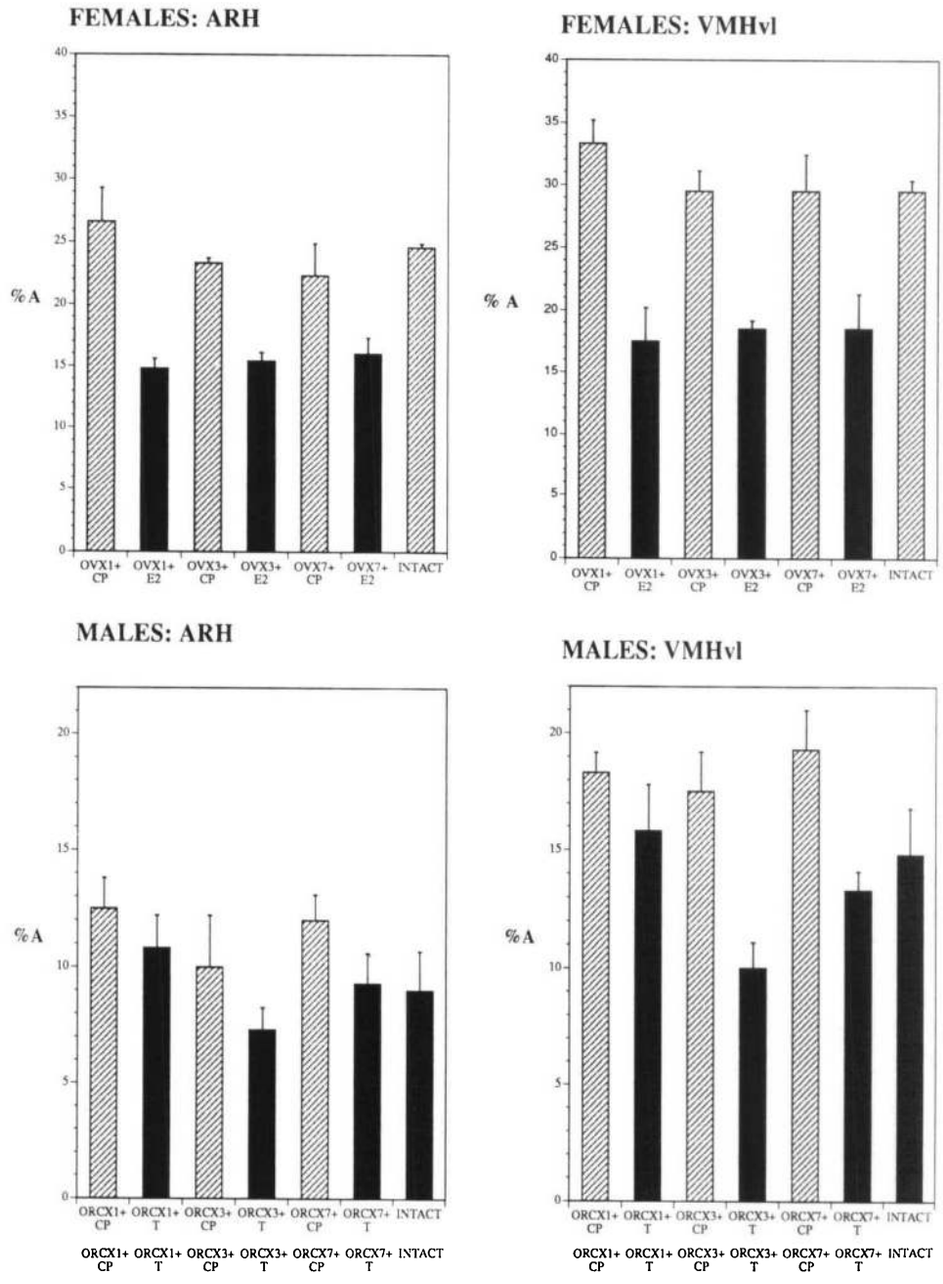

Fig. 2. Regulation of ER mRNA in the ARH and VMHvl

Comparison of hybridization signals for ER mRNA in the ARH and VMHvl of female (*top*) and male (*bottom*) rats. Females were intact (estrous) or were ovariectomized and treated with pellets of E_2 (0.05 mg/pellet) or control pellets (CP) for 1, 3, or 7 days before death. Intact male rats were compared with animals that were orchidectomized (ORCX) and treated with pellets of T or control pellets (CP) for 1, 3, or 7 days before death. *Bars* represent mean percent absorbance values (±SEM) for each experimental group. *Solid bars* indicate animals with relatively high, and *hatched bars* indicate animals with very low or undetectable, levels of circulating sex steroid hormones. n = 4 for each experimental group.

obtained over the ARH after 8 days of exposure. ER mRNA hybridization in the VMHvl was decreased by approximately 40% after 24 h of E_2 treatment compared with that in intact females and decreased by 50% relative to that in ovariectomized control animals that received CP treatment for 24 h. Reduced levels of ER mRNA hybridization were maintained with additional E_2 treatment for up to 7 days. As was found in the ARH, no significant differences were detected between levels of ER mRNA hybridization in the VMHvl in intact females relative to ovariectomized control animals (Fig. 2). The density of silver grains overlying individual cells in the emulsion-dipped material was consistently lower in animals treated with E_2 relative to that in intact or ovariectomized control animals (Fig. 3, A and B).

Males

Treatment of orchidectomized male rats with T also reduced levels of ER mRNA hybridization in the VMHvl,

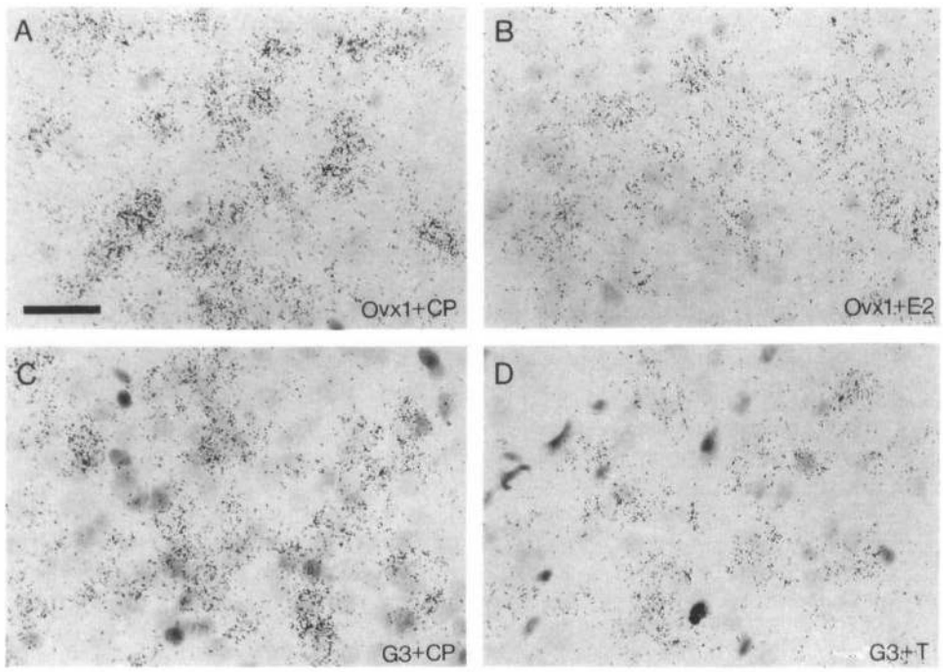

Fig. 3. Influence of Sex Steroids on Cellular Labeling for ER mRNA in the ARH

High power photomicrographs showing the relative intensity of labeling over cells in the ARH in an ovariectomized female rat treated for 1 day with either a control pellet (Ovx1 + CP; A) or E_2 (Ovx1 + E_2; B) and over cells in the ARH of an orchidectomized male rat treated for 3 days with either a control pellet (G3 + CP; C) or T (G3 + T; D). *Scale bar* = 20 μm.

although the changes appeared to be less robust and followed a different time course than that seen in females. Treatment of orchidectomized rats with T for 24 h did not result in noticeable changes in ER mRNA levels in the VMHvl, but 3-day treatments decreased ER mRNA hybridization signals over the VMHvl by approximately 40% relative to those in gonadectomized animals that received CP for 3 days. After an additional 4 days of T treatment the hybridization signals remained lower than those of gonadectomized animals treated with CP for the same period.

As was found for females, the density of silver grains over ER mRNA-containing cells within the VMHvl in dipped autoradiograms appeared to be decreased after 3 days of hormone treatment relative to that in control animals processed in parallel, indicating that the changes in ER mRNA hybridization detected on film are due to fluctuations in cellular levels of specific message. Relative to intact males, orchidectomy resulted in a significant increase in ER mRNA hybridization only after 7 days. T-treated male rats had levels of ER mRNA hybridization similar to those of intact males.

A comparable trend toward down-regulation by T was seen for ER mRNA hybridization in the ARH, and as was the case for VMHvl, the greatest apparent decrease was found in animals treated with T for 3 days (Fig. 3, C and D). However, this decrease did not achieve statistical significance, perhaps because of the small sample size and relatively low levels of ER mRNA hybridization detected in the ARH of males.

DISCUSSION

Our *in situ* hybridization data clearly indicate that circulating sex steroids down-regulate ER mRNA in the ARH and VMHvl of the rat brain. These findings are in good agreement with previous immunohistochemical results (Refs. 1 and 33–35; but see also Ref. 6) and suggest further that hormonally induced changes in ER levels may be due to regulation at the biosynthetic level. E_2 has been shown to down-regulate levels of both ER mRNA and protein in MCF-7 cancer cells (40–43) as well as in rat uterus (45). However, estrogen treatment was reported to cause a significant increase in ER mRNA in T47-D cells, similar to that found in rat liver and pituitary (42, 45), suggesting that the influence of E_2 on ER gene expression is both cell and tissue specific. Although the overall regulatory pattern was similar for the ARH and the VMHvl, the percent change observed for the VMHvl was somewhat less, possibly because of what appeared to be a greater abundance of ER mRNA in these neurons.

It is somewhat more difficult to correlate the present findings with previous estrogen binding data. Treatment of female rats with E_2 increases [^{3}H]E_2 binding in brain tissue, as determined by nuclear exchange assay (37–39), which is consistent with increased levels of nuclear binding detected during proestrus in cycling animals (38). Similar results have been obtained for male rats (36). These hormone-induced increases in estrogen binding, however, are generally not interpreted to reflect

an increase in cellular levels of the ER, but, rather, indicate an increase in the number of receptors translocated to the nuclear compartment (14) or an increase in the affinity of hormone-receptor complexes for chromatin and other nuclear components (12, 15). Thus, estrogen not only activates available receptors through specific binding, which increases the affinity of the receptor for nuclear acceptor sites, but also appears to cause a decrease in the biosynthesis of the receptor. These two regulatory influences may, therefore, represent dual molecular levels at which sex steroids alter the responsiveness of a target cell to hormonal modulation.

The ARH and VMHvl both contain high densities of cells that express the progesterone receptor (47), and cells have been identified in the ARH and VMH of the guinea pig brain that appear to cocontain ER and progesterone receptor immunoreactivities (48, 49). Thus, the possibility that the removal of progesterone by ovariectomy may have influenced ER expression cannot be ruled out. Progesterone appears to decrease estrogen binding in the uterus (50, 51), but conflicting results have been reported for the brain (52, 53), and no significant changes have been observed in nuclear ER immunostaining in the ARH and VMHvl after progesterone treatment (48). In addition, the progestin R5020 partially blocked the inhibitory effects of E_2 on ER mRNA and protein in MCF-7 cells (42), indicating that complex regulatory interactions may exist between these two hormone receptors. Nevertheless, it appears unlikely that the increases in ER mRNA within ARH and VMHvl neurons observed in ovariectomized rats are due solely to the removal of an inhibitory influence mediated by the progesterone receptor.

In male rats, circulating sex steroids also appear to down-regulate ER mRNA in the VMHvl, but with a different time course than that observed in females. Whereas in female rats a significant decrease in ER mRNA was detected by 24 h of E_2 treatment, comparable changes were not detected in male animals until after 3 days of treatment with T. The interpretation of this finding is complicated by the fact that T may be converted to E_2 by the enzyme aromatase (54). Therefore, if E_2 is the chief steroid responsible for suppressing levels of ER mRNA, it follows that this suppression would be attenuated in male animals, in which intraneuronal T must first be converted to E_2 before affecting ER expression. Consistent with this interpretation, we were unable to detect significant changes in ER mRNA in the ARH, which has low aromatase activity (55). Alternatively, since the VMHvl contains large numbers of cells that contain androgen receptors (6, 7, 56), T may affect ER mRNA levels through an androgen receptor-mediated mechanism that displays different kinetics. The nonaromatizable androgen dihydrotestosterone has been shown to down-regulate ER mRNA and protein in ZR-75–1 cancer cells (44). This finding raises the possibility that the inhibitory influence of T treatment on ER mRNA in the VMHvl may be at least partially mediated by the androgen receptor.

Although the observed difference in the time course of ER regulation in male and female rats may be due to differences in the actions of androgen and estrogen on ER gene expression, it may also reflect a sex difference in the responsiveness of neurons in the hypothalamus to hormonal activation. For instance, the induction of lordosis behavior by estrogen (57) and the stimulation of progestin receptors (58) are both greater in female rats than in males. Moreover, $[^3H]E_2$ binding to nuclear chromatin is attenuated in male rats (59), which may represent either a lower capacity for receptor binding or a significant difference in nuclear factors that influence the affinity of the activated receptor for DNA. Alternatively, our results may reflect a sexual dimorphism in levels of ER expression within the ARH and VMHvl. Although the present study was not designed to address this question, we consistently found higher levels of ER mRNA hybridization in female animals. This finding is consistent with earlier histochemical observations (7) as well as the results of hormone binding (58, 68) and autoradiographic studies (69).

In both male and female rats, the densities of silver grains overlying individual cells in the emulsion-dipped material were consistently lower in hormone-treated animals than in gonadectomized controls, indicating that the observed changes in ER mRNA hybridization signals detected on the film autoradiograms are due to changes in the cellular levels of ER mRNA. A recent preliminary report of a 42% decrease in the density of ER hybridization over individual neurons in the VMHvl of female rats that occurs within 18 h after treatment with E_2 benzoate (60) supports this conclusion.

Taken together with the findings of earlier immunohistochemical studies, the present results suggest that circulating E_2 negatively autoregulates the biosynthesis of its own receptor. However, at present, it is not known whether ER biosynthesis in the ARH and VMHvl, as inferred from mRNA levels, is primarily regulated at the transcriptional or posttranscriptional level, since the extremely low levels of ER mRNA in the hypothalamus preclude direct measurements of gene transcription and mRNA stability using current methodology. An estrogen response element (11, 61–63) in the 5′ flanking region of the rat ER gene has yet to be identified, and estrogen appears to act primarily at the posttranscriptional level to suppress steady state levels of ER mRNA in MCF-7 cells, although in the same cells a transient suppression of ER expression that followed a nearly 2-fold induction of gene transcription was observed (43). In view of the cell- and tissue-specific regulation demonstrated for the ER, the possibility that estrogen treatment induces the expression of a second *trans*-acting regulatory factor, present in hypothalamic neurons that suppresses ER gene expression also remains a viable explanation for the observed down-regulation of ER mRNA in the ARH and VMHvl.

The ARH and VMH are key components of a sexually differentiated forebrain circuitry in the rat brain that has been proposed to mediate many of the hormonal regulatory influences on reproductive function (46, 64, 65).

The strong inputs to the ARH and VMHvl from other forebrain regions that contain large numbers of ER-containing neurons (64, 66) and observed changes in estrogen binding in the medial basal hypothalamus after deafferentation (67, 70) suggest that these two nuclei may receive transneuronal regulatory influences as well as direct hormonal feedback mediated by the ER. Thus, negative autoregulation of ER gene expression may represent an important molecular mechanism by which estrogen modulates its functional impact on widespread, but interconnected, hormone-sensitive circuits involved in the control of neuroendocrine physiology and behavior.

MATERIALS AND METHODS

Animals

Seven groups of female and seven groups of male adult Sprague-Dawley rats were studied. The vaginal cycles of the female rats were monitored daily between 0800–1100 h; only those females exhibiting at least two consecutive 4-day cycles were included in this study. In Exp I, intact female (estrous) rats were perfused together with female rats that had been ovariectomized and immediately given sc pellets (0.5 mg) of 17β-estradiol (E_2; Innovative Research of America, Toledo, OH) or a control pellet 1, 3, or 7 days before death (four animals per group). In Exp II, intact male rats were perfused together with male rats that had been orchidectomized and immediately give sc pellets (25 mg) of T (Innovative Research of America) or a control pellet 1, 3, or 7 days before death (four animals per group). These experimental treatments provide rapidly achieved and constant supraphysiological doses of hormone at each time point studied. They are designed to optimize the detection of hormonal effects on ER gene regulation *in vivo* and do not attempt to replicate a particular aspect of the normal physiological state of intact animals.

Tissue Preparation

Each animal was deeply anesthetized with tribromoethanol, and a 1- to 2-ml blood sample was taken from the left jugular vein immediately before perfusion. After a brief saline rinse, each animal was perfused transcardially with ice-cold 4% paraformaldehyde in 0.1 M borate buffer at pH 9.5, after which the brains were quickly removed and postfixed overnight at 4 C in the same fixative containing 20% sucrose. The brains were randomized and numerically coded before histological processing. Twenty-micron thick frozen sections (at a frequency of one in four) through the ARH and VMH of each brain were collected in chilled 0.02 M potassium-PBS that contained 0.25% paraformaldehyde (pH 7.4), and mounted onto gelatin-subbed poly-L-lysine-coated microscope slides from the same buffer, but lacking the paraformaldehyde. After a 30-min proteinase-K digestion (10 μg/ml; 37 C) and acetylation (0.0025%; room temperature), the sections were dehydrated in ascending alcohols and dried under vacuum for at least 2 h.

In Situ Hybridization

The hybridization protocol used in the present study essentially follows that of Cox *et al.* (71). SP6 polymerase was used to transcribe ^{35}S-labeled antisense cRNA riboprobes from an 850-basepair (bp) *Xba*I-*Eco*RI fragment subcloned from the full-length ER cDNA (72) generously supplied by Dr. Muramatsu of the University of Tokyo. The resulting riboprobe is complimentary to the mRNA encoding the entire steroid-binding domain of the rat ER as well as to approximately 75 bp of the 3′ untranslated region (see Fig. 1 of Ref. 7). The radiolabeled cRNA probe was purified by passing the transcription reaction solution over a P60 column, and 12 200-μl fractions were collected and counted using a scintillation counter. The leading fraction (typically the fourth) was identified by its extremely high specific activity (1.5–4.3 $\times$ 10^6 dpm/μl). The purified cRNA probe was heated at 65 C for 5 min with 500 μg/ml yeast tRNA and 50 μM dithiothreitol in water before being diluted to an activity of 1.5 $\times$ 10^7 dpm/ml with hybridization buffer containing 50% formamide, 0.25 M sodium chloride, 1 $\times$ Denhardt's solution, and 10% dextran sulfate. This hybridization solution was pipetted onto the sections (70 μl/slide), covered with a glass coverslip, and sealed with DPX (Gallard and Schleisenger, Carle Place, NY) before incubation for 20 h at 60 C. This hybridization time was chosen because we previously determined by RoT (RNA over time; see Ref. 71) analysis that under our hybridization conditions the ER probe appears to saturate hybridizable ER mRNA by at least 18 h. After hybridization the slides were washed four times (10 min each) in 4 $\times$ SSC before RNase digestion (20 μg/ml; 30 min at 37 C), rinsed at room temperature in decreasing concentrations of SSC that contained 1 mM dithiothreitol (DDT) (2-, 1-, and 0.5-fold; 10 min each) to a final stringency of 0.1 $\times$ SSC at 75 C for 30 min. After dehydration in increasing alcohols, the sections were exposed to DuPont Cronex x-ray films (DuPont, Wilmington, DE) for 4 and 8 days before being dipped in NTB-2 liquid emulsion. The dipped autoradiograms were developed 14 days later with Kodak D-19 developer (Eastman Kodak, Rochester, NY), and the sections were counterstained with thionin through the emulsion.

Several control experiments were carried out to test the specificity of both the hybridization method and the ER probe. First, sections were incubated, as described above, with hybridization solution containing 1.5 $\times$ 10^7 dpm/ml sense strand (not complementary to ER mRNA) probe synthesized using T7 polymerase to transcribe the coding strand of the DNA insert. Second, hybridization was also attempted on sections that had been pretreated with RNase (20 μg/ml; 30 min at 37 C). Third, sections through the hypothalamus were incubated overnight with an excess of unlabeled ER probe or unlabeled probes for androgen and mineralocorticoid receptor mRNA (7, 73) and subsequently incubated with a similar concentration of the labeled ER probe. Posthybridization treatments were then carried out on these control sections, as described above, to a final stringency of 0.1 $\times$ SSC at 75 C. In addition, the thermal stability of the ER hybrids was assessed by taking seven adjacent series of sections through the hypothalamus that had been hybridized as described above and rinsing one series each in 0.1 $\times$ SSC at 65, 75, 80, 85, 90, and 100 C (7). Finally, the detailed distribution of ER mRNA-containing cells throughout the brain was compared with that of mRNA-containing cells labeled using probes directed toward 15 different neuronal messages, including those encoding tyrosine hydroxylase, preprocholecystokinin, preprotachykinin, and the androgen, mineralocorticoid, and glucocorticoid receptors (7, 21, 26, 73).

Quantification of Autoradiograms

The integrated optical density of the autoradiographic images on Cronex x-ray film was measured, as described previously (74), using a Joyce-Loebl Magiscan image analysis system (marketed by Nikon) with a graphics program developed by Brittain (75). The film was illuminated with a ChromaPro 45 light source, which provided even illumination, and the image was obtained with a VST Labs (Ann Arbor, MI) model SC501 CCD camera.

The integrated optical density of the autoradiographic images on Cronex x-ray film over the ARH and VMHvl at the same levels from each brain were measured. The integrated optical density over five separate square areas surrounding

the ARH and VMHvl that did not contain specific hybridization were measured and used to calculate mean background levels, which were subtracted from the integrated optical density measurements of signals over the ARH and VMHvl. To facilitate comparisons between measurements, the integrated optical density measurements were converted to percent absorbance (a scale from 0–100%). Autoradiographic standards were prepared by thoroughly mixing brain paste (homogenized fresh brain tissue plus 0.1 M Tris buffer) with varying amounts of ^{35}S-labeled UTP. The standard mixtures were added to centrifuge tubes, frozen with powdered dry ice, and sectioned at 20 μm. Sections from each of 12 standards (plus one blank that lacked isotope) were mounted onto slides and stored desiccated at −70 C. One series each of the standards was exposed to the x-ray films along with experimental material. The integrated optical density of an interactively defined region over each standard was measured; these measurements confirmed the linearity of the film's responsiveness as well as the consistency of signal detection across films (four films per experiment). In order that the optical densities of the autoradiographic images over the ARH and VMHvl were all within the linear range of the standard values, measurements over the ARH (males and females) were made from films exposed for 8 days, as were those for the VMHvl in males, but 4-day exposures were required for the VMHvl in female animals.

A two-way analysis of variance was used to test for significant differences in levels of ER mRNA hybridization among the treatment groups in each experiment, and a *post-hoc* Duncan's multiple range test was applied to identify significant differences between individual treatment groups. $P < 0.05$ or less was defined as significant.

Hormone Assays

Blood samples taken immediately before perfusion were collected into precooled heparinized tubes. Plasma was separated by centrifugation and stored at −20 C. Plasma levels of E_2 (females) were measured by RIA, as described previously (76). Plasma levels of T (males) were measured in antibody-coated tubes (Diagnostic Products Corp., Los Angeles, CA). All samples for E_2 and T were each run in a single assay, with an intraassay variation of less than 4%, and the lower limits of detection were 2 pg/ml and 0.2 ng/ml for E_2 and T, respectively.

Acknowledgments

We thank Dr. M. Muramatsu for generously providing the ER cDNA, and Dr. Catherine Rivier for contributing the data on plasma E_2 and T concentrations. We also wish to thank Dr. M. Schalling for helpful suggestions, as well as Dr. L. W. Swanson for valuable discussions and in whose laboratory this work was carried out. We are grateful to Mr. Kris Trulock for photographic assistance, and Mr. David Warren and Ms. Lori Hennings for secretarial help.

Received April 20, 1990. Revision received December 11, 1990. Accepted December 28, 1990.

Address requests for reprints to: R. B. Simerly, Division of Neuroscience, Oregon Regional Primate Research Center, 505 NW 185th Avenue, Beaverton, Oregon 97006.

This work was supported by the Howard Hughes Medical Institute.

REFERENCES

1. Cintra A, Fuxe K, Härfstrand A, Agnati LF, Miller LS, Greene JL, Gustafsson J-Å 1986 On the cellular localization and distribution of estrogen receptors in the rat tel- and diencephalon using monoclonal antibodies to human estrogen receptor. Neurochem Int 8:587–595
2. Fuxe K, Cintra A, Agnati LF, Härfstrand A, Wikstrom A-C, Okret S, Zoli M, Miller LS, Greene JL, Gustafsson J-Å 1987 Studies on the cellular localization and distribution of glucocorticoid receptor and estrogen receptor immunoreactivity in the central nervous system of the rat and their relationship to the monoaminergic and peptidergic neurons of the brain. J Steroid Biochem 27:159–170
3. Pfaff D, Keiner M 1973 Atlas of estradiol-concentrating cells in the central nervous system of the female rat. J Comp Neurol 1:121–158
4. Press MF, Greene GL 1988 Immunocytochemical localization of estrogen and progesterone receptors. In: DeLellis RA (ed) Advances in Immunohistochemistry. Raven Press, New York, pp 341–361
5. Rainbow TC, Parsons B, MacLusky NJ, McEwen BS 1982 Estradiol receptor levels in rat hypothalamic and limbic nuclei. J Neurosci 2:1439–1445
6. Sar M, Parikh I 1986 Immunohistochemical localization of estrogen receptor in rat brain, pituitary and uterus with monoclonal antibodies. J Steroid Biochem 24:497–503
7. Simerly RB, Chang C, Muramatsu M, Swanson LW 1990 Distribution of androgen and estrogen receptor mRNA-containing cells in the rat brain: an *in situ* hybridization study. J Comp Neurol 294:76–95
8. Stumpf WE, Sar M, Keefer DA 1975 Atlas of estrogen target cells in rat brain. In: Stumpf WE, Grant LD (eds) Anatomical Neuroendocrinology. Karger, Basel, pp 104–119
9. Fink G 1988 Oestrogen and progesterone interactions in the control of gonadotrophin and prolactin secretion. J Steroid Biochem 30:169–178
10. Pfaff DW, Schwartz-Giblin S 1988 Cellular mechanisms of female reproductive behaviors. In: Knobil E, Neill J (eds) The Physiology of Reproduction. Raven Press, New York, pp 1487–1568
11. Evans RM 1988 The steroid and thyroid hormone receptor superfamily. Science 240:889–895
12. Gorski J, Welshons WV, Sakai D, Hansen J, Walent J, Kassis J, Shull J, Stack G, Campen C 1986 Evolution of a model of estrogen action. Recent Prog Horm Res 42:297–329
13. King RJB 1987 Structure and function of steroid receptors. J Endocrinol 114:341–349
14. McEwen BS, Biegon A, Davis PG, Krey LC, Luine VN, McGinnis MY, Paden CM, Parsons B, Rainbow TC 1982 Steroid hormones: humoral signals which alter brain cell properties and functions. Recent Prog Horm Res 38:41–92
15. Walters MR 1985 Steroid hormone receptors and the nucleus. Endocr Rev 6:2–543
16. Alexander MJ, Dobner PR, Miller MA, Bullock BP, Dorsa DM, Leeman SE 1989 Estrogen induces neurotensin/neuromedin N messenger ribonucleic acid in a preoptic nucleus essential for the preovulatory surge of luteinizing hormone in the rat. Endocrinology 125:2111–2117
17. Baldino Jr F, Fitzpatrick-McElligott S, O'Kane TM, Gozes I 1988 Hormonal regulation of somatostatin messenger RNA. Synapse 2:317–325
18. Harlan RE 1988 Regulation of neuropeptide gene expression by steroid hormones. Mol Neurobiol 2:183–200
19. Roberts JL, Dutlow CM, Jakubowski M, Blum M, Millar RP 1989 Estradiol stimulates preoptic area-anterior hypothalamic proGnRH-GAP gene expression in ovariectomized rats. Mol Brain Res 6:127–134
20. Romano GJ, Mobbs CV, Howells RD, Pfaff DW 1989 Estrogen regulation of proenkephalin gene expression in the ventromedial hypothalamus of the rat: temporal qualities and synergism with progesterone. Mol Brain Res 5:58
21. Simerly RB, Young BJ, Capozza MA, Swanson LW 1989 Estrogen differentially regulates neuropeptide gene

expression in a sexually dimorphic olfactory pathway. Proc Natl Acad Sci USA 86:4766–4770
22. Van Tol HHM, Bolwerk ELM, Liu B, Burbach JPH 1988 Oxytocin and vasopressin gene expression in the hypothalamo-neurohypophyseal system of the rat during the estrous cycle, pregnancy, and lactation. Endocrinology 122:945–949
23. Wilcox JN, Roberts JL 1985 Estrogen decreases rat hypothalamic proopiomelanocortin messenger ribonucleic acid levels. Endocrinology 117:2392–2396
24. Blum M, McEwen BS, Roberts JL 1987 Transcriptional analysis of tyrosine hydroxylase gene expression in the tuberoinfundibular dopaminergic neurons of the rat arcuate nucleus after estrogen treatment. J Biol Chem 262:817–821
25. Morrell JI, Rosenthal MF, McCabe JT, Harrington CA, Chikaraishi DM, Pfaff DW 1989 Tyrosine hydroxylase mRNA in the neurons of the tuberoinfundibular region and zona incerta examined after gonadal steroid hormone treatment. Mol Endocrinol 3:1426–1433
26. Simerly RB 1989 Hormonal control of the development and regulation of tyrosine hydroxylase expression within a sexually dimorphic population of dopaminergic cells in the hypothalamus. Mol Brain Res 6:297–310
27. Wallis CJ, Luttge WG 1980 Influence of estrogen and progesterone on glutamic acid decarboxylase activity in discrete regions of rat brain. J Neurochem 34:609–613
28. Hoffman GE, Lee W-S, Attardi B, Yann V, Fitzsimmons MD 1990 Luteinizing hormone-releasing hormone neurons express c-*fos* antigen after steroid activation. Endocrinology 126:1736–1741
29. Insel TR 1990 Regional induction of c-*fos*-like protein in rat brain after estradiol treatment. Endocrinology 126:1849–1853
30. Lingham RB, Stancel GM, Loose-Mitchell DS 1988 Estrogen regulation of epidermal growth factor receptor messenger ribonucleic acid. Mol Endocrinol 2:230–235
31. Loose-Mitchell DS, Chiappetta C, Stancel GM 1988 Estrogen regulation of *c-fos* messenger ribonucleic acid. Mol Endocrinol 2:946–949
32. Romano GJ, Krust A, Pfaff DW 1989 Expression and estrogen regulation of progesterone receptor mRNA in neurons of the mediobasal hypothalamus: an *in situ* hybridization study. Mol Endocrinol 3:1295–1300
33. Blaustein JD, Turcotte JC 1989 Estrogen receptor-immunostaining of neuronal cytoplasmic processes as well as cell nuclei in guinea pig brain. Brain Res 495:75–82
34. DonCarlos LL, Monroy E, Malik K, Morrell JI, Estrogen receptor-immunoreactivity in the forebrain of the female guinea pig: distribution and effect of steroid hormone treatment. 15th Annual Meeting of the Society for Neuroscience, Washington DC, 1989, p 627 (Abstract)
35. Warembourg M, Jolivet A, Milgrom E 1989 Immunohistochemical evidence of the presence of estrogen and progesterone receptors in the same neurons of the guinea pig hypothalamus and preoptic area. Brain Res 480:1–15
36. Krey LC, Lieberburg I, Roy E, McEwen BS 1979 Oestradiol plus receptor complexes in the brain and anterior pituitary gland: quantitation and neuroendocrine significance. J Steroid Biochem 11:279–284
37. Lustig RH, Mobbs CV, Bradlow HL, McEwen BS, Pfaff DW 1989 Differential effects of estradiol and 16α-hydroxyestrone on pituitary and preoptic estrogen receptor regulation. Endocrinology 125:2701–2709
38. McGinnis MY, Krey LC, MacLusky NJ, McEwen BS 1981 Steroid receptor levels in intact and ovariectomized estrogen-treated rats: an examination of quantitative, temporal and endocrine factors influencing the efficacy of an estradiol stimulus. Neuroendocrinology 33:158–165
39. Wise PM, Parsons B 1984 Nuclear estradiol and cytosol progestin receptor concentrations in the brain and the pituitary gland and sexual behavior in ovariectomized estradiol-treated middle-aged rats. Endocrinology 115:810–816
40. Berkenstam A, Glaumann H, Martin M, Gustafsson J-Å, Norstedt G 1989 Hormonal regulation of estrogen receptor messenger ribonucleic acid in $T47D_{co}$ and MCF-7 breast cancer cells. Mol Endocrinol 3:22–28
41. Ree AH, Landmark BF, Eskild W, Levy FO, Lahooti H, Jahnsen T, Aakvaag A, Hansson V 1989 Autologous down-regulation of messenger ribonucleic acid and protein levels for estrogen receptors in MCF-7 cells: an inverse correlation to progesterone receptor levels. Endocrinology 124:2577–2583
42. Read LD, Greene GL, Katzenellenbogen BS 1989 Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonists, and growth factors. Mol Endocrinol 3:295–304
43. Saceda M, Lippman ME, Chambon P, Lindsey RL, Ponglikitmongkol M, Puente M, Martin MB 1988 Regulation of the estrogen receptor in MCF-7 cells by estradiol. Mol Endocrinol 2:1157–1162
44. Poulin R, Simard J, Labrie C, Petitclerc L, Dumont M, Lagace L, Labrie F 1989 Down-regulation of estrogen receptors by androgens in the ZR-75–1 human breast cancer cell line. Endocrinology 125:392–399
45. Shupnik MA, Gordon MS, Chin WW 1989 Tissue-specific regulation of rat estrogen receptor mRNAs. Mol Endocrinol 3:660–665
46. Swanson LW 1987 The hypothalamus. In: Björklund A, Hökfelt T, Swanson LW (eds) Handbook of Chemical Neuroanatomy, vol 5, part 1. Elsevier, Amsterdam, pp 1–124
47. Warembourg M 1978 Uptake of ^{3}H labeled synthetic progestin by rat brain and pituitary. A radioautography study. Neurosci Lett 9:329–332
48. Blaustein JD, Turcotte JC 1989 Estradiol-induced progestin receptor immunoreactivity is found only in estrogen receptor-immunoreactive cells in guinea pig brain. Neuroendocrinology 49:454–461
49. Warembourg M, Jolivet A, Milgrom E 1989 Immunohistochemical evidence of the presence of estrogen and progesterone receptors in the same neurons of the guinea pig hypothalamus and preoptic area. Brain Res 480:1–15
50. Okulicz WD, Evans RW, Leavitt WW 1981 Progesterone regulation of estrogen receptor in the rat uterus: a primary inhibitory influence on the nuclear fraction. Steroids 37:463–470
51. Okulicz WC, Evans RW, Leavitt WW 1981 Progesterone regulation of the occupied form of nuclear estrogen receptors. Science 213:1503–1505
52. Barraclough CA, Camp P, Weiland N, Akabori A 1986 Stimulatory versus inhibitory effects of progesterone on estrogen-induced phasic LH and prolactin secretion correlated with estrogen nuclear and progestin cytosol receptor concentrations in brain and pituitary gland. Neuroendocrinology 42:6–14
53. Blaustein JD, Brown TJ 1984 Progesterone decreases the concentration of hypothalamic and anterior pituitary estrogen receptors in ovariectomized rats. Brain Res 304:225–236
54. Celotti F, Massa R, Martini L 1979 Metabolism of sex steroids in the central nervous system. In: DeGroot LJ (ed) Endocrinology. Grum and Stratten, New York, vol 1:41–53
55. Roselli CE, Horton LE, Resko JA 1985 Distribution and regulation of aromatase activity in the rat hypothalamus and limbic system. Endocrinology 117:2471–2477
56. Sar M, Stumpf WE 1975 Distribution of androgen-concentrating neurons in rat brain. In: Stumpf WE, Grant LD (eds) Anatomical Neuroendocrinology. Karger, Basel, pp 120–133
57. Harlan RE, Shivers BD, Pfaff DW 1984 Lordosis as a sexually dimorphic neural function. Prog Brain Res 61:239–255
58. MacLusky NJ, Brown TJ 1989 Control of gonadal steroid receptor levels in the developing brain. In: Lakoski JM,

Perez-Polo JR, Rassin DK (eds) Neural Control of Reproductive Function. Liss, New York, pp 45–59
59. Olsen KL, Whalen RE 1980 Sexual differentiation of the brain: effects on mating behavior and [^{3}H]-estradiol binding by hypothalamic chromatin in rats. Biol Reprod 22:1068–1079
60. Lauber AH, Romano GJ, Mobbs CV, Chambon P, Pfaff DW, Estradiol regulation of estrogen receptor mRNA in rat hypothalamus. 15th Annual Meeting of the Society for Neuroscience, Washington DC, 1989, p 984 (Abstract)
61. Klein-Hitpass L, Tsai SY, Greene GL, Clark JH, Tsai M-J, O'Malley BW 1989 Specific binding of estrogen receptor to the estrogen response element. Mol Cell Biol 9:43–49
62. Peale FV, Ludwig LB, Zain S, Hilf R, Bambara RA 1988 Properties of a high-affinity DNA binding site for estrogen receptor. Proc Natl Acad Sci USA 85:1038–1042
63. Shapiro DJ, Barton MC, McKearin DM, Chang T-C, Lew D, Blume J, Nielsen DA, Gould L 1989 Estrogen regulation of gene transcription and mRNA stability. Recent Prog Horm Res 45:29–64
64. Cottingham SL, Pfaff D 1986 Interconnectedness of steroid hormone-binding neurons: existence and implications. In: Ganten J, Pfaff D (eds) Current Topics in Neuroendocrinology. Springer-Verlag, Berlin, vol 7:223–249
65. Simerly RB, Swanson LW 1988 Projections of the medial preoptic nucleus: a *Phaseolus vulgaris* leucoagglutinin anterograde tract-tracing study in the rat. J Comp Neurol 270:209–242
66. Chronwall BM 1985 Anatomy and physiology of the neuroendocrine arcuate nucleus. Peptides 6:1–11
67. Carrillo AJ, Sheridan PJ 1980 Estrogen receptors in the medial basal hypothalamus of the rat following complete hypothalamic deafferentation. Brain Res 186:157–164
68. Brown TJ, Hochberg RB, Zielinski JE, MacLusky NJ 1088 Regional sex differences in cell nuclear estrogen-binding capacity in the rat hypothalamus and preoptic area. Endocrinology 123:1761–1770
69. Kurth S, Akesson TR, Micevych PE, Bloch GJ 1990 Sex difference in estratiol (E_2)-concentrating cells within specific components of the rat medial preoptic area (MPOA). Soc Neurosci Abstr 16:764
70. McGinnis MY, Phelps CP, Nance DM, McEwen BS 1982 Changes in estrogen and progestin receptor binding resulting from retrochiasmatic knife cuts. Physiol Behav 29:225–229
71. Cox KH, DeLeon DV, Angerer LM, Angerer RC 1984 Detection of mRNAs in sea urchin embryos by *in situ* hybridization using asymmetric RNA probes. Dev Biol 101:485–502
72. Koike S, Sakai M, Muramatsu M 1987 Molecular cloning and characterization of rat estrogen receptor cDNA. Nucleic Acids Res 15:2499–2523
73. Arriza JL, Simerly RB, Swanson LW, Evans RM 1988 The neuronal mineralocorticoid receptor as a mediator of glucocorticoid response. Neuron 1:887–900
74. Swanson LW, Simmons DM 1989 Differential steroid hormone and neural influences on peptide mRNA levels in CRH cells of the paraventricular nucleus: a hybridization histochemical study in the rat. J Comp Neurol 285:413–435
75. Brittain DA 1988 The efferent connections of the infralimbic cortex in the rat. PhD Thesis, Department of Neurosciences, University of California-San Diego
76. Anderson D, Hopper B, Lasley B, Yen SSC 1976 A simple method for the assay of eight steroids in small volumes of plasma. Steroids 28:179–184