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Estrogen Receptor Immunoreactivity in Rat Brain: Rapid Effects of Estradiol Injection*

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ABSTRACT

The existence of cytoplasmic estrogen receptor immunoreactivity (ER-IR) has been reported in the brain using three different antibodies raised against different epitopes on the ER protein. With each antibody, the darkest reaction product was seen within cell nuclei, but cytoplasmic ER-IR was also observed with each antibody in most neuroantaomical areas. We have reported previously that an injection of estradiol causes a rapid decrease in ER-IR in ovariectomized guinea pigs when the H 222 estrogen receptor antibody was used. In extending this work to rats, we used three different ER antibodies to determine if this decrease in ER-IR is likely to be due to down-regulation of the receptor by estradiol or a decrease in the ability of some antibodies to bind to their particular epitopes on the receptor. Estradiol injection in ovariectomized rats caused a rapid (within 20 min), nearly total loss of cytoplasmic ER-IR when the H 222 antibody was used to visualize the receptor. This decrease did not appear to be due to movement of the receptors to the cell nucleus, as cell nuclear ER-IR also decreased. When other antibodies were used, the extent of loss and the pattern of immunostaining were greatly influenced by the particular antibody. Extensive loss of cytoplasmic and cell nuclear ER-IR was seen when an antiserum against the hinge region of the receptor was used. However, when an antiserum against the N-terminus of the ER was used, a decrease was seen in cytoplasmic ER-IR, but little or no decrease was observed in cell nuclear ER-IR. Because loss of cell nuclear ER-IR was not seen with all of the antibodies, these results suggest that the dramatic decrease in cell nuclear ER-IR seen immediately after estradiol injection is due at least in part to a conformational change in the receptor. This, in turn, may impede association with particular antibodies during the immunocytochemical procedure. (*Endocrinology* **132:** 1218–1224, 1993)

AS IN OTHER types of cells (1), estrogen receptors (ERs) are believed to mediate many cellular responses to estradiol in neurons (2). The neuroanatomical distribution of ERs with the same characteristics as those present in peripheral reproductive tissues has been described in neuronal populations using autoradiography (3, 4) and immunocytochemistry (5–8). Although it had been believed that the unoccupied receptor was a cytoplasmic protein that was translocated to cell nuclei upon binding to ligand (9), it has also been suggested that the unoccupied receptor is loosely associated with cell nuclei (10, 11), and the unoccupied receptor that appears in cytosol after tissue disruption might be an artifact of tissue disruption. There is abundant evidence to support each of these interpretations.

While we have confirmed the cell nucleus as the intracellular site of densest ER immunoreactivity (ER-IR) in ovariectomized guinea pig brain, we have also reported the presence of ER-IR in perikaryal cytoplasm and cytoplasmic processes of many neurons with an ER-IR nucleus (6, 12, 13). Despite numerous papers reporting the lack of cytoplasmic ER-IR in the brain (5, 8, 14–19, 20–22), ER immunostaining has now been reported in neuronal cytoplasmic processes in guinea pigs (6, 7, 12, 13), rats (13), musk shrews (23), opossums (24), and ferrets (25).

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We have recently confirmed the existence of ER-IR in perikaryal cytoplasm and cytoplasmic processes in rat brain using three different antibodies directed at three independent sites on the ER (13). In addition to the H 222 monoclonal antibody directed against the ligand-binding site of the receptor (26), we used a polyclonal antiserum against the "hinge" region between the steroid-binding domain and the DNA-binding domain of the rat ER (27) and a polyclonal antiserum raised against the 21-amino acid N-terminus of the rat ER (Greene, G., personal communication).

We have shown previously that an estradiol injection results in the rapid loss of cytoplasmic ER-IR in the brain (6, 12). We suggested that this was not likely to be due to translocation of the receptors to the cell nuclei, because cell nuclear ER-IR decreased as well. We concluded that the decrease in ER-IR was not due to down-regulation of the ER protein, because it occurred rapidly, within 1 h (12) of estradiol injection. Rather, we have suggested that the decreased ER-IR is a limitation of the antibody used in previous work, as the H 222 antibody is directed against the ligand-binding site of the ER (26). In the present experiment, we examined the rapid effects of injection of estradiol on ER-IR in the rat brain visualized with these three antibodies.

Materials and Methods

Animals

Adult CD strain female rats, weighing 225–300 g at the time of perfusion, were obtained from Charles River Breeding Laboratories (Wilmington, MA) and group-housed in stainless steel cages. A 14-h light, 10-h dark cycle was maintained, with lights on from 2100–1100 h. Seven to 10 days before perfusion, all rats were ovariectomized

bilaterally through a midventral incision under methohexital sodium anesthetic (Brevital, Eli Lilly Co., Indianapolis, IN; 52 mg/kg BW). Animals were killed during the light phase of the illumination cycle. Food (Purina laboratory chow, Ralston-Purina, St. Louis, MO) and water were freely available. Twenty minutes before perfusion, rats were injected sc either with 50 μg 17β-estradiol dissolved in 0.1 ml 10% ethanol vehicle (n = 6) or the ethanol-water vehicle (n = 5).

Perfusion

Animals were deeply anesthetized with a combination of sodium pentobarbital and chloral hydrate. Five thousand units of sodium heparin dissolved in 1 ml saline were injected directly into the left ventricle. The thoracic aorta and posterior vena cava were clamped, the right atrium and apex of the heart were cut, and a cannula was inserted through the left ventricle into the aorta. Physiological saline (15 ml) preceded the flow of fixative containing 4% paraformaldehyde in a 0.1 M sodium phosphate buffer (pH 7.2) with (n = 3 for vehicle; n = 3 for estradiol-injected) or without (n = 2 for vehicle; n = 3 for estradiol injected) 0.1% glutaraldehyde. Perfusion pressure was maintained at approximately 100 mm Hg, and flow rate was maintained at approximately 25 ml/min for 10 min. After removal from the cranium, brains were blocked and stored in a 20% sucrose-0.1 M sodium phosphate buffer (pH 7.2) before cutting 40-μm coronal sections with a freezing microtome. Sections were then placed into cryoprotectant (28) for at least 1 h before the start of the immunocytochemical procedure.

Immunocytochemistry

All sections from all animals were run simultaneously to eliminate the possibility of variability among immunocytochemical runs. Free-floating sections were rinsed three times for 5 min each time to remove the cryoprotectant. They were then treated with 1% sodium borohydride in Tris-buffered saline (TBS) for 10 min to remove residual aldehydes (29). After four rinses, sections were incubated for 20 min in TBS containing 1% hydrogen peroxide, 20% normal goat serum, and 1% BSA to deplete endogenous peroxidase activity and decrease nonspecific staining, respectively.

The sections were then incubated for approximately 40 h in one of three primary antibodies diluted in TBS containing 1% goat serum, 0.1% gelatin, 0.5% Triton X-100, and 0.02% sodium azide, pH 7.6, at 4 C. The primary antibodies used to immunostain ERs in sections from rats included H 222, which is a rat monoclonal antibody directed against the ligand-binding domain of the human ER (26) (Abbott Laboratories, North Chicago, IL; 2–5 μg/ml); ER 21 (gift of Geoffrey Greene, University of Chicago), which is a rabbit polyclonal antiserum directed against the N-terminus of the ER (5 μg/ml); or ER 715 (27), which is a rabbit polyclonal antiserum made against amino acids 270–284, as predicted by the cloned cDNA sequence (30) in the hinge region of the ER (0.2 μg/ml). We have reported previously the results of control studies in which the ER 21 and ER 715 antisera were preadsorbed with the appropriate peptide fragments (13), and the H 222 antibody was preadsorbed with a cellular extract enriched in ER (12), or in which primary antibodies were omitted from the immunocytochemical procedure. After three washes (5 min each) in the same buffer, the sections were incubated for 90 min at room temperature in secondary antiserum (biotinylated goat antirat immunoglobulins or biotinylated goat antirabbit immunoglobulins at a concentration of 5 μg/ml; Jackson ImmunoResearch, Inc., West Grove, PA) in the same buffer as the primary antibody. After two washes with and one without Triton X-100 (5 min each), the sections were incubated for 90 min in the streptavidin reagent (Vectastain Elite Kit, Vector Laboratories, Burlingame, CA). After two washes in TBS containing sodium azide and Triton X-100 and one wash in TBS, the sections were incubated with diaminobenzidine (0.05% in TBS) in the presence of hydrogen peroxide (0.05%) for 10 min and then rinsed in TBS.

Sections were mounted on subbed slides and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). Reaction product was examined at ×200 magnification within matched sections, including the medial preoptic area, the ventrolateral-ventromedial hypothalamus and the adjacent area lateral to it, and the medial amygdala. The intensity of ER-IR within an intracellular compartment was rated blindly on a subjective 0–4 scale. Ratings were made independently on cell nuclei, perikaryal cytoplasm, and cytoplasmic processes. A rating of 4 was given to a section demonstrating the highest intensity of reaction product within a particular subcellular compartment of a particular neuroanatomical area, and a rating of 0 was given for the absence of reaction product associated with a particular subcellular compartment in that neuroanatomical area. We rated on a scale in which the greatest level of reaction product seen in a particular neuroanatomical area was considered 4, rather than on a constant scale. This is because the immunocytochemical procedure was optimized for the hypothalamus and preoptic area, and we have shown that optimal immunocytochemical results for different neuroanatomical areas may require different fixation conditions (31). Because of the similarity in ratings between animals perfused with or without the addition of 0.1% glutaraldehyde, the groups of animals receiving each fixative were combined.

Statistical contrasts were made using the Mann-Whitney U test for independent samples, and results were considered statistically significant if $P < 0.05$. The limitations of immunocytochemical procedures prohibit quantitative contrasts among different antibodies or antisera. Furthermore, contrasts cannot be made among different neuroanatomical areas, because different brain regions may require different fixation conditions for optimal immunostaining (31).

Results

H 222 directed against steroid-binding domain

As described previously (13), optimal H 222 immunostaining is seen in rats when a fixative of 4% paraformaldehyde and 0.4% glutaraldehyde was used. However, to contrast the ER-IR seen with other antibodies in the same animals, we evaluated H222-IR in the less optimal fixation of 4% paraformaldehyde with or without 0.1% glutaraldehyde. In vehicle-injected rats, extensive ER-IR was seen in the medial preoptic area and amygdala, although with slightly higher background than when 0.4% glutaraldehyde was included in the fixative (13). As expected with this fixative, a high background was seen within the medial hypothalamus, making it impossible to evaluate H 222-IR in that region in rats. Within the preoptic area, numerous cells had ER-IR nuclei; and many of these cells also had ER-IR in the perikaryal cytoplasm, and a smaller subset of these had ER-IR extensively associated with cytoplasmic processes (Fig. 1). As we reported previously, while many ER-IR cells were observed in the amygdala, few of these had ER-IR cytoplasmic processes, and ER-IR in perikaryal cytoplasm was inconsistent. Therefore, only ratings of cell nuclei are reported for this area.

Estradiol injection resulted in virtually total elimination of ER-IR in cytoplasmic processes [$U(5,6) = 1$; $P < 0.01$] and perikaryal cytoplasm [$U(5,6) = 1$; $P < 0.01$] and a large decrease in cell nuclear ER-IR [$U(5,6) = 0$; $P < 0.01$] in the medial preoptic area (Table 1).

ER 715 directed against the hinge region

Using the ER 715 antibody, the level of cell nuclear ER-IR in the medial preoptic area was similar to that seen with the H222 antibody, but reaction product in perikarya and processes was less intense (Fig. 1 and Table 1). High levels of reaction product were also observed in cell nuclei and cytoplasm in the ventrolateral-ventromedial hypothalamic area (Fig. 2) and in cell nuclei in the medial amygdala as well.

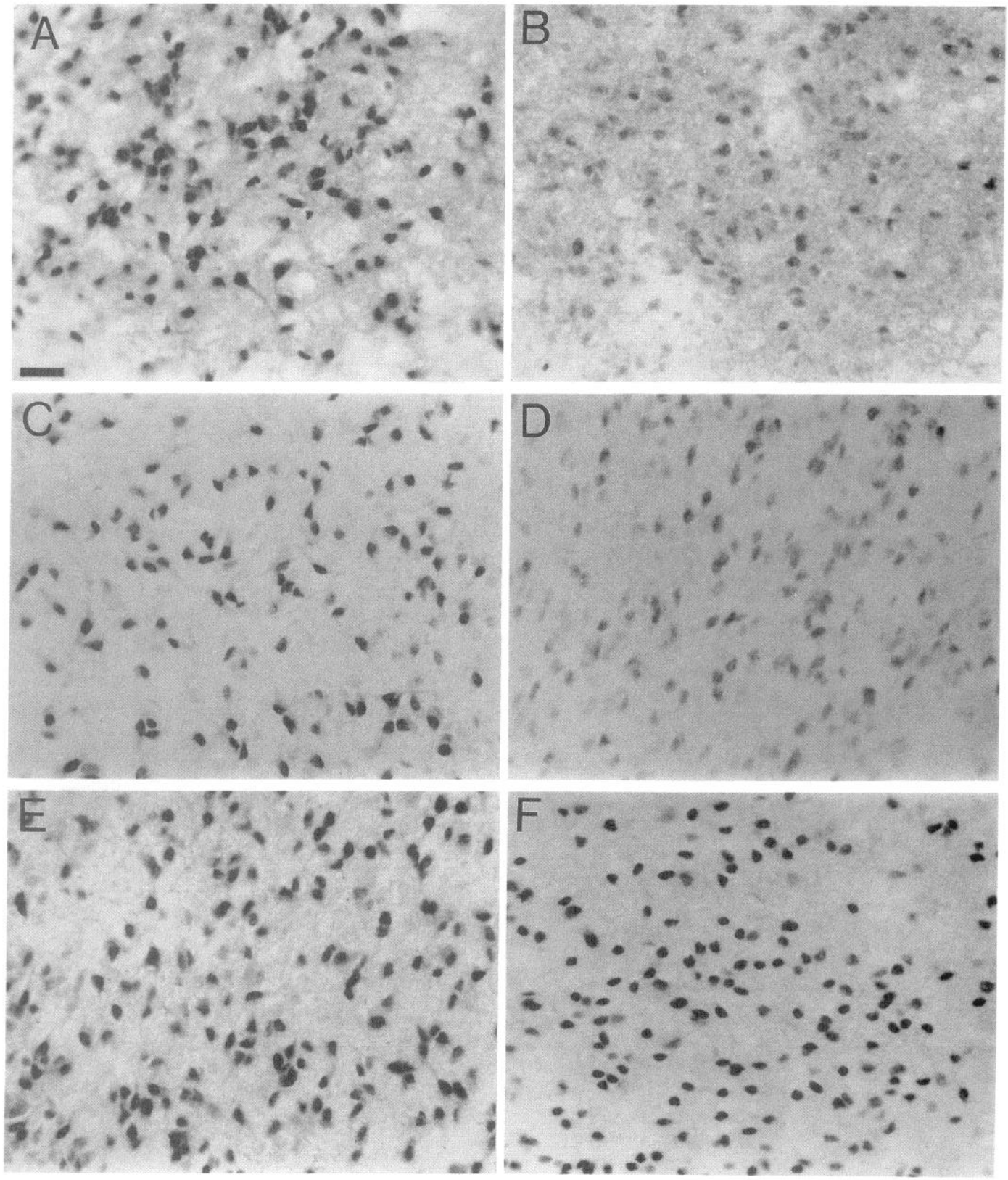

FIG. 1. ER-IR in the medial preoptic area of (A, C, and E) of estradiol-uninjected ovariectomized rats or (B, D, and F) estradiol-injected ovariectomized rats (50 μg; 20 min before perfusion) immunostained with the following antibodies: H 222 (A and B), ER 715 (C and D, and ER 21 (E and F). Magnification *bars*, 50 μm.

TABLE 1. Ratings of ER-IR in three neuroanatomical areas after vehicle or estradiol injection (20 min before perfusion)

	H222			ER715			ER21		
	Nuclei	Perikaryal cytoplasm	Processes	Nuclei	Perikaryal cytoplasm	Processes	Nuclei	Perikaryal cytoplasm	Processes
Medial preoptic area									
Vehicle									
Mean	3.8	3.5	2.7	3.6	1.9	1.2	3.7	2.6	2.1
SEM	0.12	0.32	0.37	0.24	0.10	0.74	0.20	0.24	0.33
n	5	5	5	5	5	5	5	5	5
Estradiol									
Mean	2.3	0.5	0.3	1.5	0	0	3.2	1.2	0.2
SEM	0.25	0.50	0.33	0.18	0	0	0.28	0.38	0.17
n	6	6	6	6	6	6	6	6	6
Ventrolateral-ventromedial hypothalamic area									
Vehicle									
Mean	—[a]	—	—	3.6	2.8	2.4	3.7	3.4	2.6
SEM	—	—	—	0.10	0.49	0.68	0.20	0.24	0.75
n	—	—	—	5	5	5	5	5	5
Estradiol									
Mean	—	—	—	1.4	0.33	0	3.6	2.7	0.8
SEM	—	—	—	0.42	0.33	0	0.20	0.61	0.54
n	—	—	—	6	6	6	6	6	6
Amygdala									
Vehicle									
Mean	3.2	—	—	2.6	—	—	2.4	—	—
SEM	0.37	—	—	0.37	—	—	0.29	—	—
n	5	—	—	5	—	—	5	—	—
Estradiol									
Mean	2.3	—	—	0.5	—	—	2.6	—	—
SEM	0.17	—	—	0.32	—	—	0.2	—	—
n	6	—	—	6	—	—	6	—	—

[a] See text for explanation of ratings that are not reported.

Estradiol injection eliminated all reaction product in cytoplasmic processes [*e.g.* medial preoptic area; $U(5,6) = 0$; $P < 0.01$] and perikaryal cytoplasm [$U(5,6) = 0$; $P < 0.01$]. It also caused a dramatic decrease in cell nuclear ER-IR in all three neuroanatomical areas [*e.g.* medial preoptic area; $U(5,6) = 0$; $P < 0.01$].

ER 21 directed against N-terminus

Using the ER 21 antibody, the levels of cell nuclear ER-IR in the medial preoptic area (Fig. 1 and Table 1) and ventrolateral-ventromedial hypothalamic area (Fig. 2) were similar to those seen with the H 222 and ER 715 antibodies. Perikarya and processes also showed high levels of ER-IR. High levels of reaction product were found in cell nuclei in the medial amygdala as well. With this antibody, we also occasionally observed reaction product with an appearance distinctly different from that seen with the other two antibodies. This immunostaining of fine fibers in some animals was observed inconsistently and was present in both vehicle- and estradiol-injected rats (*e.g.* reaction product in fine fibers in Fig. 2D).

Estradiol injection had less of an effect when the ER 21 antibody was used than when the ER 715 or H 222 antibodies were used. When ER-IR was visualized using the ER 21 antiserum, the only statistically significant effects of estradiol injection were seen on cytoplasmic processes [$U(5,6) = 0.5$; $P < 0.01$] and perikaryal cytoplasm [$U(5,6) = 2$; $P < 0.02$] in the medial preoptic area, and the decrease appeared more variable than that observed with the ER 715 and H 222 antibodies. No effect was seen in the level of reaction product in cell nuclei from the medial preoptic area, ventrolateral-ventromedial hypothalamic area, or amygdala.

Discussion

We have reported the existence of cytoplasmic ERs in brains of a variety of species. In guinea pigs, we have shown that an injection of estradiol decreases ER-IR within 1 h (12). This did not appear to be due to movement of the ER from cytoplasm to cell nucleus, because cell nuclear ER-IR decreased too. We attributed the decrease in ER-IR to the specificity of the antibody used; the H 222 monoclonal antibody is directed against the ligand-binding domain of the ER. This interpretation was somewhat difficult to reconcile with the fact that the H 222 antibody was directed against the occupied form of the ER, and the antibody binds to occupied ERs *in vitro* (32). The recent development of two additional antisera directed against distinct epitopes on the ER protein have allowed the further appraisal of this interpretation.

We used a dose of estradiol greatly in excess of the dose required to saturate ERs in rats after sc injection (33) to ensure rapid occupation of ERs by estradiol, and we immunocytochemically stained ERs with three antibodies in sections cut from the same animals. Rats were perfused within 20 min of injection; a latency before extensive, if any, down-regulation of ERs is likely to occur.

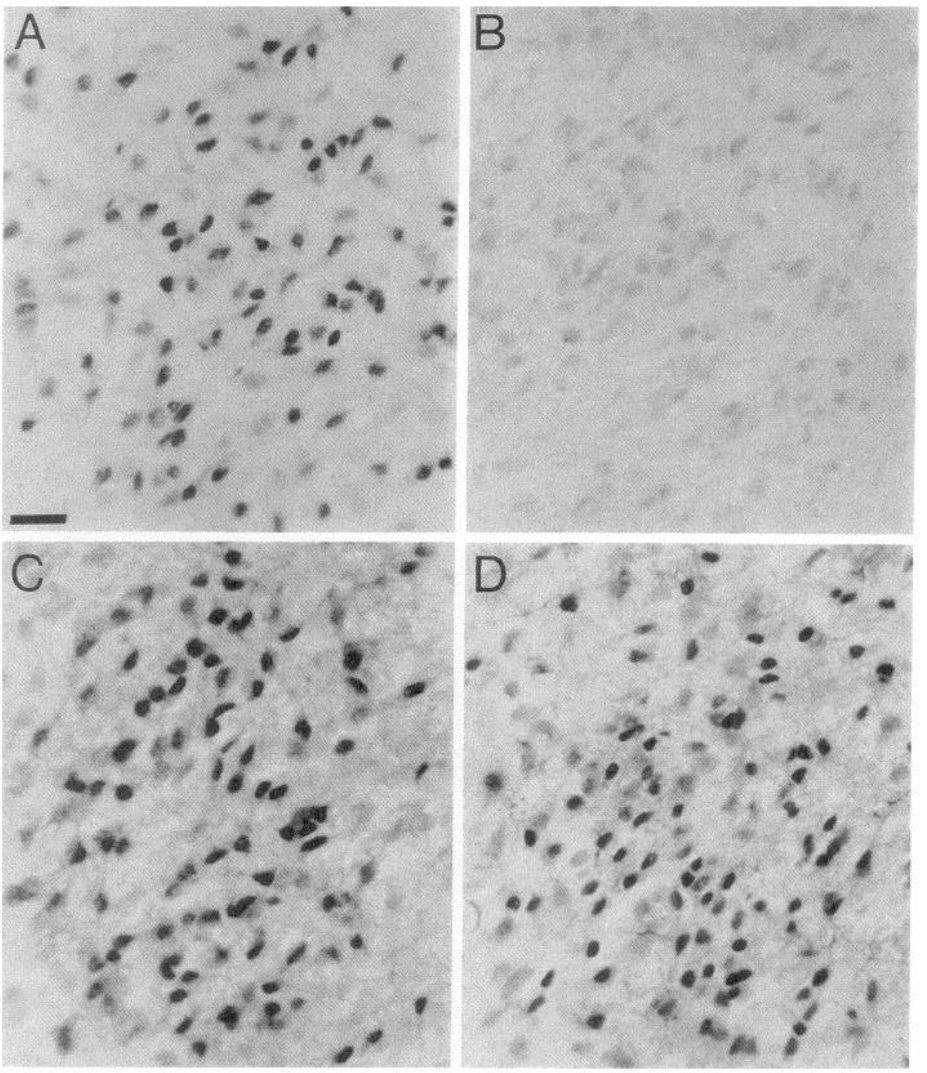

FIG. 2. ER-IR in the ventrolateral aspect of the ventromedial hypothalamus of estradiol-uninjected ovariectomized rats (A and C) or estradiol-injected rats (B and D; 50 μg, 20 min before perfusion) immunostained with the following antibodies: ER 715 (A and B) and ER 21 (C and D). Magnification *bars* = 50 μm.

While the intensity of ER-IR in cell nuclei decreased with two of the antibodies, no apparent decrease was observed when the ER 21 antibody was used. This suggests then that the concentration of ER present in cell nuclei was not decreased by estradiol injection. There are several possible explanations for these results. 1) It has been suggested that estradiol binding to ER causes dimerization of the receptors (34–36) and dissociation of heat shock protein 90 (37). These types of changes in the receptor may result in the epitopes to which the H 222 and ER 715 antibodies bind, becoming less accessible to these antibodies (38, 39). 2) Association of the receptors with estradiol could result in the receptors binding within the cell nucleus, such that the ligand-binding region and hinge region become less accessible to antibody. 3) Binding of the receptor to DNA may, in turn, alter the conformation of particular domains of the ER (40). While the fate of cell nuclear ER-IR after estradiol injection cannot be determined definitively with immunocytochemical techniques, the results strongly suggest that the nuclear ERs have not been down-regulated by the estradiol injection within the 20-min latency.

In other experiments in which the H 222 antibody was used, decreases in cell nuclear ER-IR were seen within 1 h of estradiol injection (5, 6, 12); however, in an experiment in which a different antibody was used (JS 34/32) (14), an increase was seen. Unfortunately, no information is available on the epitope of the JS 34/32 antibody that was used in that experiment, but we suggest that this discrepancy may be due to that antibody having an epitope that becomes more accessible to antibody after estradiol binds to the receptor. That study, however, provides further support that the early effect of estradiol on cell nuclear ER-IR is not due to down-regulation of ER.

Estradiol does, in fact, down-regulate ERs in the brain; ER mRNA in the hypothalamus decreases within 2 h of estradiol injection (41, 42), and ERs decrease markedly after sustained elevation of estradiol levels (43). In some cell lines, ER levels are down-regulated as early as 3 h after treatment (44). Therefore, it seems quite likely that a secondary effect of estradiol action in at least some brain areas is down-regula-

tion of the receptor, but the immediate decrease seen in cell nuclear ER-IR after estradiol injection is unlikely to be due to down-regulation of receptors.

The fate of cytoplasmic ER-IR after estradiol injection is of interest. We cannot exclude the possibility that some of the extranuclear ER-IR in the vehicle-injected rats is due to diffusion during perfusion of unoccupied receptors loosely bound by cell nuclei (10, 11). Consequently, we cannot exclude the possibility that a portion of the decrease in extranuclear ER-IR seen after estradiol injection is due to the occupied receptor being more tightly bound by cell nuclei (10, 11). Nevertheless, the fact that ER-IR in cytoplasmic processes disappears after estradiol injection regardless of the antibody used suggests that at least a portion of the decrease in cytoplasmic ER-IR is due to either movement of the receptors or association with an organelle in which the receptors are less accessible to the antibody. It is possible that some of the perikaryal cytoplasmic ER-IR could represent receptors that shuttle between cytoplasm and nucleus (45); however, it is unlikely that those in distal cytoplasmic processes (at least up to 500 μm from the cell nucleus) could move into cell nuclei, at least within the 20-min time course of this experiment.

In summary, besides demonstrating that ER-IR is observable in cell nuclei after estradiol injection using some antibodies, but not others, this experiment demonstrates that caution should be exercised when interpreting the results of immunocytochemical experiments on ER under conditions in which estradiol is present.

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