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Localization of androgen receptors and estrogen receptors in the same cells of the songbird brain

(immunocytochemistry/autoradiography/canary)

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ABSTRACT **Estrogens and androgens each have unique effects but act together for the neural differentiation and control of sexual behaviors in male vertebrates, such as the canary. The neuronal basis for these synergistic effects is elusive because the spatial relation between estrogen target cells and androgen target cells is unknown. This study localized estrogen receptor (ER)-containing cells by using immunocytochemistry and androgen receptor (AR)-containing cells by using autoradiography in the same sections of the male canary brain. Three cell types, those containing only ER, those containing only AR, and those containing both ER and AR, were found in tissue-specific frequencies. The midbrain nucleus intercollicularis exhibited the highest number of cells expressing both ER and AR, whereas ER and AR are expressed only in disjunctive cell populations in the forebrain nucleus hyperstriatalis ventrale, pars caudale. Synergistic effects of androgens and estrogens for the neural behavorial control could result from cells containing both ER and AR (intracellular) and from neural circuits containing ER and AR in different cells (intercellular).**

The effects of steroid hormones on the development and induction of sexual behaviors correlate with changes in mRNA and protein synthesis in brain areas involved in the neural control of those behaviors (1). The gene-regulatory effects of estrogens and androgens are due to their binding to intracellularly located estrogen receptors (ER) and androgen receptors (AR), respectively, and subsequent receptor genome interactions within cells of the target area (2, 3). Within a target area of estrogens or androgens, only a fraction of all cells contain AR or ER and are therefore the primary loci of steroids' gene-regulatory effects. The present study investigates whether ER and AR are expressed in the same cells or in different cells in the same brain areas of the canary, a songbird species. The songbird brain contains androgen- or estrogen-sensitive brain nuclei involved in the control of complex behaviors, such as male courtship and vocal behavior (4–7).

MATERIALS AND METHODS

Autoradiographic Localization of AR-Containing Cells. For the localization of AR-containing cells, the cellular uptake of the androgen 5α-dihydrotestosterone (5α-DHT) was studied in autoradiographic procedures. The androgen 5α-DHT is not convertible to estrogens. Six adult male canaries (*Serinus canaria*) in breeding condition were anesthetized with Equithesin, castrated, and injected with 5α-[^{3}H]DHT [5 ng (3 μCi) per gram of body weight (New England Nuclear, no. NET544), specific activity = 190 Ci/mmol (1 Ci = 37 GBq)] dissolved in 70% ethanol 48 hr after castration. The brains were removed 90 min later and frozen over liquid nitrogen. Brains were cut into 10-μm parasagittal sections with a cryostat at −20°C, and the sections were mounted onto photoemulsion-coated slides (Kodak NTB 3) under a safelight. Control sections were mounted onto gelatin-coated slides and directly immunostained with the estrogen receptor antibody H222SPγ (Abbott) as noted below (see Fig. 1*D*).

For autoradiography, slides were stored in lightproof boxes containing Drierite at −70°C for 12–18 months. Sections were then fixed with phosphate-buffered 4% paraformaldehyde; the slides were developed with buffered Kodak D19 and photochemically fixed with Unifix (Eastman Kodak). After the autoradiography, every third section was Nissl-stained without further immunostaining for histological identification of labeled brain areas.

A cell was considered to be tritium-labeled if it had 5 times more silver grains over its soma than adjacent cell-sized areas of neuropil (8). The distribution of tritium-labeled cells in the canary brain was area-specific and similar in all animals, except in the animal used to control for binding saturability. To control for the saturability of binding, one canary was injected with a 100-fold excess of unlabeled 5α-DHT 15 min before the injection of 5α-[^{3}H]DHT. The tritium labeling was widely blocked in this competition experiment; a few cells were still labeled in the nucleus hyperstriatalis ventrale, pars caudale (HVC), nucleus magnocellularis anterioris, and nucleus nervi hypoglassi (see areas 1, 4, and 16 of Fig. 2). To control for the specificity of binding, two canaries were treated with estradiol benzoate in Silastic tube implants (Corning; 0.76 mm i.d. × 1.65 mm o.d., 7 mm long) immediately after castration. The circulating concentration of 17β-estradiol (E_2) of these two birds was measured by radioimmunoassay (9) in blood samples taken prior to the injection of the 5α-[^{3}H]DHT. The E_2 titers in the estrogen-treated animals were 8 and 7.5 ng/ml of plasma and thus 6 times higher than the mean E_2 titer of intact males in breeding condition (9) (the interassay variance of the laboratory of H.-R. Güttinger for E_2 was 13.6%). The tritium labeling was unaffected in the estrogen-implanted animals and is therefore hormone-specific. In agreement with this control, ER show a high affinity for E_2 and a very low affinity for 5α-DHT in songbird brain tissue (10).

In summary, the tritium labeling was area-specific, nuclear, saturable, and hormone-specific. These characteristics of the tritium binding and the fact that 5α-DHT is only very slowly metabolized to other androgens (11) indicate a specific binding of 5α-DHT to AR.

Comparison Between AR Autoradiography and ER Immunocytochemistry. After autoradiography, sections were re-

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Abbreviations: AR, androgen receptor(s); ER, estrogen receptor(s); 5α-DHT, 5α-dihydrotestosterone; E_2, 17β-estradiol; ICO, nucleus intercollicularis; PVN, nucleus paraventricularis magnocellularis; HVC, nucleus hyperstriatalis ventrale, pars caudale.

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acted with the monoclonal ER antibody H222Spγ (Abbott) in indirect immunocytochemical procedures for the localization of ER-containing cells as described elsewhere in detail (12). As a modification to this protocol, a double-bridge technique was used to enhance the staining (13). Briefly, sections were treated with 0.1% Triton X-100 in 25 mM phosphate buffer containing 0.9% NaCl (PBS) for 30 min. Sections were first incubated with 2% goat serum in PBS (30 min), then in the ER antibody H222Spγ (1 μg/ml in PBS) (Abbott), then in the second antibody (goat anti-rat IgG, 1:50 in PBS) (Sigma), then in the third antibody (rat clonoPAP, 1:100 in PBS; Sternberger-Meyer, Garretsville, MD), and then again with the second antibody followed by the third antibody. Incubation times were 1 hr at room temperature for each antibody followed by a 30-min wash in PBS. After the last wash, the sections were incubated for 10 min in the chromagen containing 0.03% diaminobenzidine and 0.01% hydrogen peroxide for visualization of the immunoproduct. Antibody-labeled cells had darkly stained cell nuclei (see Fig. 1). For analysis of the tritium labeling and the total cell number, immunostained sections were counterstained with methyl green.

In sections processed for both autoradiography and immunocytochemistry, antibody-labeled cells and tritium-labeled cells were counted under high power on a Zeiss microscope. Five sections (taken at 50-μm intervals) of each brain area that contained labeled cells of each of three animals were analyzed. In each area section, the percentage of a cell type was calculated based on the total number of labeled cells in this area (Fig. 3).

To estimate the frequency of labeled cells relative to the total cell number, all labeled cells were counted in 10 sections of the midbrain nucleus intercollicularis (ICO) as described above. In the areas defined by the distribution of labeled cells, all Nissl-stained cells with a visible nucleus were counted under high power with an ocular grid on a Zeiss microscope. The percentage of labeled cells among Nissl-stained cells was calculated for each section. The total number of labeled cells of this area was estimated from the number of labeled cells per area-section and the intersection distances (50 μm) for each animal. Given are the median, minimum, and maximum values of all animals.

RESULTS

In each animal, except the control that was injected with unlabeled 5α-DHT, the sections processed with both autoradiography and immunocytochemistry exhibited three types of labeled cells (Fig. 1 *A–C*): (*i*) cells labeled only with the antibody, (*ii*) cells labeled only with tritium, and (*iii*) cells double-labeled with the antibody and tritium. The frequencies of these cell types in the canary brain were area-specific (Figs. 2 and 3). Among areas that were heavily labeled with

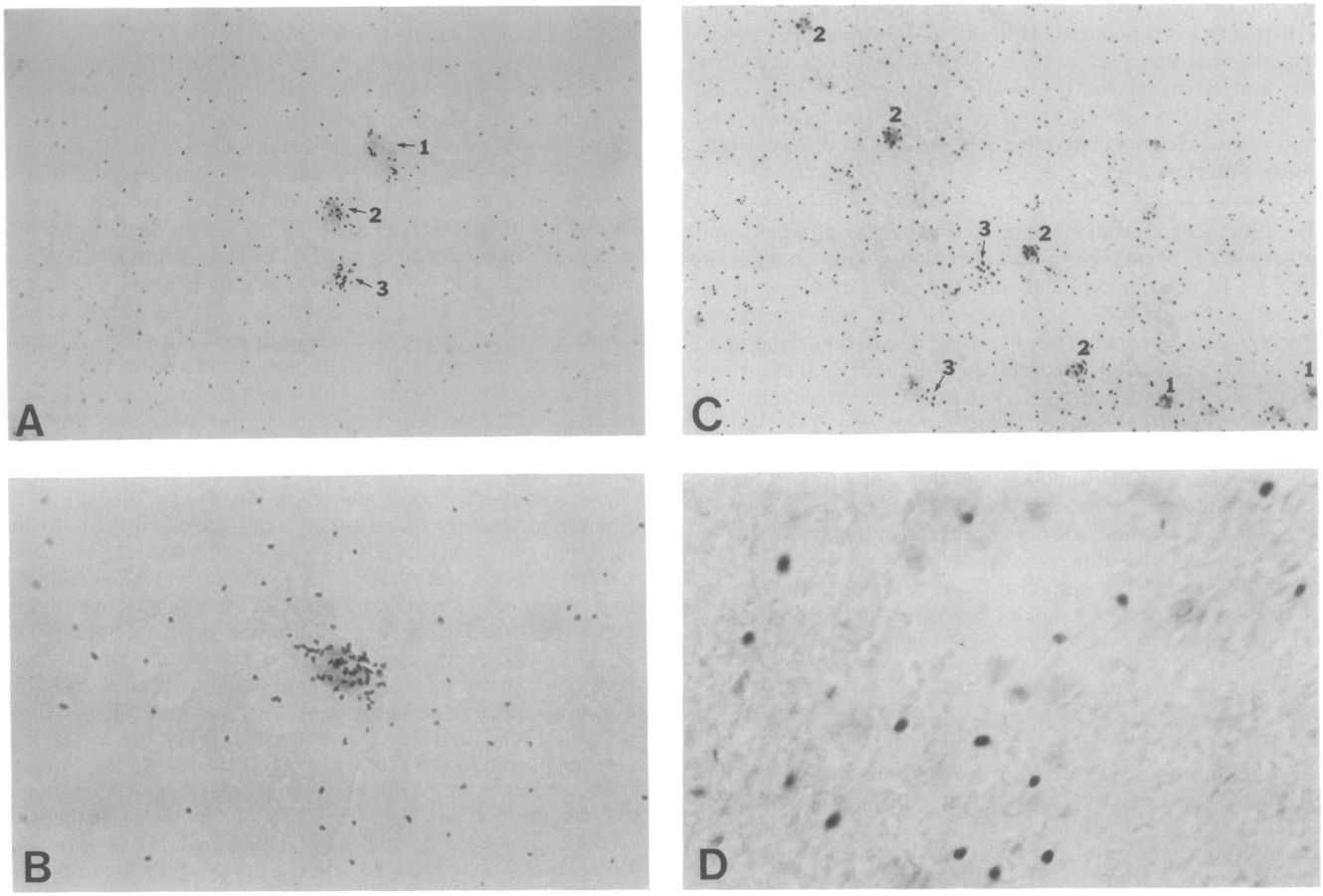

FIG. 1. Comparison between autoradiography with the tritiated androgen 5α-DHT and immunocytochemistry with the monoclonal ER antibody H222Spγ. Antibody-labeled cells have darkly labeled cell nuclei. Tritium-labeled cells have silver grains over their nuclei. (*A*) Three cells (cells 1, 2, and 3) of the midbrain ICO are shown: cell 1 is labeled only with the antibody (silver grains lay over two cells bordering the antibody-labeled cell), cell 2 is double-labeled, and cell 3 is labeled only with tritium. (×1290.) (*B*) Double-labeled ICO cell in higher magnification. (×2625.) (*C*) Cells labeled only with the antibody (cells indicated by 1), double-labeled cells (cells indicated by 2), and cells labeled only with tritium (cells indicated by 3) are interspersed in the nucleus paraventricularis magnocellularis (PVN). (×975.) Double-labeled cells contain the immunocytochemical reaction products and silver grains over their cell nuclei. (*D*) Antibody-labeled cells in a section immunostained shortly after sectioning the brain. The labeling is more intense compared to labeled cells in *A–C*, which were stained 12–18 months later, after autoradiography. (×975.) The sections in *A–D* were photographed prior to counterstaining with Nissl stain.

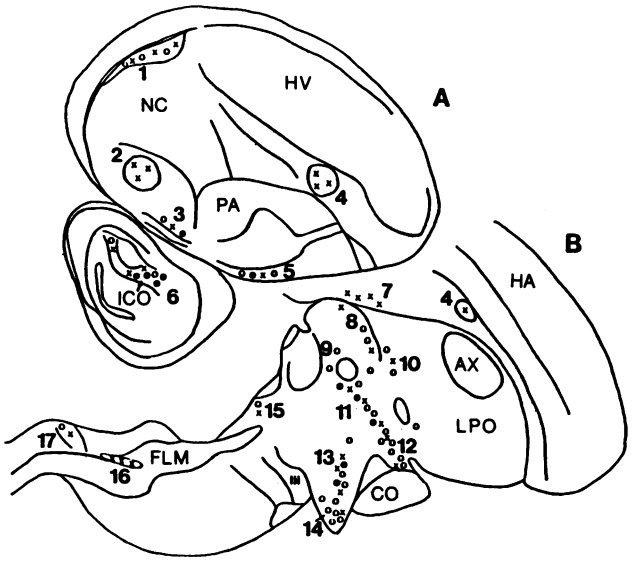

FIG. 2. Distribution of cells labeled only with tritium after the injection of 5α-[^{3}H]DHT (x, AR cells; areas 1–8 and 10–17), of cells labeled only with the ER antibody H222Spγ (○, ER cells; areas 1, 3, 5, 6, 8–15, and 17), and of cells double-labeled with tritium and antibody (●, ER-AR cells; areas 3, 5, 6, 11, and 13) in the same brain of the adult male canary. Depicted are schematic parasaggital views through the lateral (*A*) and the medial (*B*) portion of the canary brain. The distribution of ER cells, AR cells, and ER-AR cells is area-specific; certain areas contain only AR cells (areas 2, 4, 7, and 16) or only ER cells (area 9). Labeled areas: 1, HVC; 2, nucleus robustus archistriatalis; 3, above the occipito-mesencephalic fibertract; 4, nucleus magnocellularis anterioris; 5, nucleus taeniae; 6, ICO; 7, along the floor of the neostriatum; 8, lateral septum; 9, nucleus commissurae pallii; 10, nucleus of the stria terminalis around the nucleus accumbens; 11, PVN; 12, anterior preoptic area; 13, lateral hypothalamus; 14, the tuberal complex; 15, near the nucleus mesencephali nervi trigemini; 16, nucleus nervi hypoglossi; 17, nucleus solitarius. AX, area X; CO, chiasma opticum; FLM, fasciculus longitudinalis; HA, hyperstriatum accessorium; HV, hyperstriatum ventrale; LPO, lobus paraolfactorius; NC, neostriatum caudale; PA, paleostriatum augmentatum; III, nervus oculomotorius.

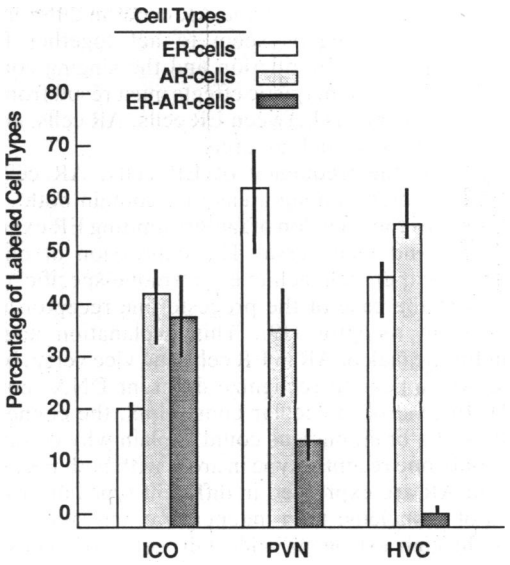

FIG. 3. Frequency of AR cells, ER cells, and ER-AR cells in the ICO, PVN, and HVC of the canary brain. Given are the median, minimum, and maximum percentages of each cell type of an area of five sections of each of three animals. The distribution of ER cells, AR cells, and ER-AR cells was significantly different between ICO, PVN, and HVC (*G*-test of independence, $P < 0.001$).

both the antibody and tritium, such as the ICO, the nucleus paraventricularis magnocellularis (PVN), the lateral hypothalamus, and the HVC, significant differences in the cellular labeling were found (Fig. 3). Cells labeled only with the antibody or only with tritium were interspersed with double-labeled cells. The ICO contained the highest number of double-labeled cells of all areas (30%), PVN and the lateral hypothalamus contained 10–15% double-labeled cells, and HVC contained few if any double-labeled cells. In the ICO area that contained antibody-, tritium-, and double-labeled cells, 30.4% (23–37%) (median and range) of the Nissl-stained cells were labeled. This area contained 2500 (2100–3000) (median and range) labeled cells.

The relative frequencies of each cell type (Fig. 3) might have been affected by the following technical difficulties: the incomplete saturation of androgen-binding sites, the thickness of the sections, the estimation of tritium-labeled cells with the rather conservative 5 times background criterion, and the loss of antigenic sites by the autoradiographic procedures. Indeed, antibody-labeled cells in sections immunostained shortly after the sectioning of the brains were darker compared to labeled cells in sections immunostained after the autoradiographic procedure (Fig. 1). Thus the number of labeled cells in sections stained directly after sectioning the brains was 30% higher than that of comparable sections immunostained 12–18 months later, after the autoradiographic procedures. However, this reduction was proportional; areas that did not react with the antibody after the autoradiography also did not exhibit antibody-labeled cells when stained directly after sectioning the brains. The estimation of the total number of labeled cells might have been affected by the above mentioned difficulties and the overcounting of labeled cells due to split cell nuclei.

DISCUSSION

Immunocytochemistry with H222Spγ identifies specifically ER-containing cells and does not crossreact with avian AR (12, 14–16). Thus, the cells of the canary brain that were immunostained with H222Spγ seem to contain ER. The characteristics of the autoradiography with 5α-[^{3}H]DHT identify tritium-labeled cells as AR-containing cells. Therefore, the combination of both techniques on the same brain sections classified four cell types in the canary brain—cells expressing (*i*) both ER and AR (ER-AR cells), (*ii*) only AR (AR cells), (*iii*) only ER (ER cells), or (*iv*) neither receptor. Because the distribution of estrogen-binding cells and androgen-binding cells in the preoptic-hypothalamic area and in the midbrain appears to be evolutionarily conserved among vertebrates (17), these cell types are to be expected also in the brain of other avian species and of reptiles and mammals. The neuronal status of the labeled cells is shown for the ER cells in the canary HVC, which are long-projection neurons (18). In the Nissl-stained sections of the present study, most of the labeled cells appear to be neurons due to their relatively large somata and their nuclear structure. However, it is difficult to classify very small labeled cells as neurons.

The distribution of ER and AR shows two different neural bases for synergistic effects of estrogens and androgens in the canary brain: first, cells that contain both ER and AR (e.g., ER-AR cells in the ICO) and, second, neural circuits that contain ER cells and AR cells (e.g., HVC). This indicates different neuronal mechanisms underlying synergistic effects of estrogens and androgens: (*i*) intracellular (ER-AR cells) and (*ii*) intercellular. In ER-AR cells synergistic effects could occur on the transcriptional level, with ER altering AR-inducible gene regulation and vice versa, or could result at the posttranscriptional level. The vocal control nucleus HVC of the canary is an example where estrogens and androgens act

synergistically but where ER and AR occur in different cells. Estrogens and androgens seem to act together for the seasonal neuronal differentiation and the singing control in the HVC (6). These synergistic effects must result from direct or indirect mechanisms between ER cells, AR cells, and cells without receptors in such an area.

Intriguingly, the frequency of ER cells, AR cells, and ER-AR cells differs among areas that contain both AR and ER. This raises the question of factors limiting ER expression in AR cells and vice versa. The expression of only one receptor type per cell achieves hormone-specific cellular responses in the case of the progesterone receptor and the glucocorticoid receptor (19). This explanation might not account for the lack of AR in ER cells and vice versa, because ER and AR appear to recognize different DNA sequences (20, 21). Instead of molecular constraints, the connectional properties of a brain nucleus could explain why certain cells express only one receptor type in areas with both ER and AR. If ER and AR are expressed in different types of projection neurons of a nucleus, then independent activation of these circuits through estrogens and androgens, respectively, is possible; e.g., the canary HVC has two main projections, the nucleus area X and the nucleus robustus, and ER and AR are found in different HVC cells. Because most of the ER cells in HVC project to area X (18), AR cells are either local interneurons or project to the nucleus robustus. In such a scope, ER-AR cells relay androgen- and estrogen-sensitive circuits, and such a cell could yield similar responses to either hormone.

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