

Neuroanatomical Specificity in the Co-Localization of Aromatase and Estrogen Receptors

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SUMMARY

The relative distributions of aromatase and of estrogen receptors were studied in the brain of the Japanese quail by a double-label immunocytochemical technique. Aromatase immunoreactive cells (ARO-ir) were found in the medial preoptic nucleus, in the septal region, and in a large cell cluster extending from the dorso-lateral aspect of the ventromedial nucleus of the hypothalamus to the tuber at the level of the nucleus inferioris hypothalami. Immunoreactive estrogen receptors (ER) were also found in each of these brain areas but their distribution was much broader and included larger parts of the preoptic, septal, and tuberal regions. In the ventromedial and tuberal hypothalamus, the majority of the ARO-ir cells (over 75%) also contained immunoreactive ER. By

contrast, very few of the ARO-ir cells were double-labeled in the preoptic area and in the septum. More than 80% of the aromatase-containing cells contained no ER in these regions. This suggests that the estrogens, which are formed centrally by aromatization of testosterone, might not exert their biological effects through binding with the classical nuclear ER. The fact that significant amounts of aromatase activity are found in synaptosomes purified by differential centrifugation and that aromatase immunoreactivity is observed at the electron microscope level in synaptic boutons suggests that aromatase might produce estrogens that act at the synaptic level as neurohormones or neuromodulators.

INTRODUCTION

The presence of aromatase activity in the brain was demonstrated in the early seventies (Naftolin, Ryan, and Petro, 1972; Naftolin et al., 1975). Since that time, a large number of studies using various experimental approaches has shown that this enzyme is critically implicated in the production of many physiological and behavioral effects of testosterone (T) in the brain. *In vitro* radioenzyme assays revealed that brain aromatase activity is regulated, especially in the preoptic area, by factors such as the sex and age of the animals as well as by their physiological condition (Balthazart,

1989). Testosterone dramatically increases preoptic aromatase activity in all species that have been investigated so far (e.g., dove: Steimer and Hutchison, 1981; Hutchison and Steimer, 1986; quail: Schumacher and Balthazart, 1986; rat: Roselli, Horton, and Resko, 1987; Roselli and Resko, 1984; monkey: Roselli and Resko, 1989). In some species, estradiol also exerts a positive feedback on the enzyme activity (dove: Hutchison and Steimer, 1986; quail: Schumacher, Alexandre, and Balthazart, 1987). Contradictory reports on this issue in rats have been published (Roselli and Resko, 1984; Roselli et al., 1987; Steimer, Arsenijevic, Aubert, and Sizonenko, 1989).

Aromatase activity in the preoptic area is necessary for the activation by T of copulatory behavior in many species. This is experimentally demonstrated by the facts that estrogens can mimic the effects of T on the behavior, aromatase inhibitors block the behavioral effects of T and also the ef-

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fects of T can be blocked by the concurrent administration of antiestrogens (for review see MacLusky, Philip, Hurlburt, and Naftolin, 1984; Balthazart, 1989). These data support the aromatization hypothesis in its strongest version which states that the restoration by T of sexual behavior in castrated males ultimately results from the interaction of estrogens derived from T aromatization with estrogen receptors. These studies have established the importance of T aromatization in the control of reproductive behavior. In addition, estrogens have important effects in the brain, including effects on neurogenesis in the fetus, synaptogenesis in the newborn, and synaptic remodeling in the adult (Naftolin et al., 1990a). They also control the secretion of gonadotrophins (Celotti, Massa, and Martini, 1979; Kalra and Kalra, 1983). Given that aromatase is present in the brain areas that regulate the release of luteinizing hormone-releasing hormone, such as the tuberal hypothalamus, estrogens formed centrally through T aromatization might play a role in the control of gonadotrophin secretion (Celotti et al., 1979), but the experimental evidence supporting this interpretation is scarce at present (Ellinwood, Hess, Roselli, Spies, and Resko, 1984; Worgul, Santen, Samojlik, Irwin, and Falvo, 1981).

By biochemical assays performed on either the entire hypothalamus or on more specific nuclei dissected by the Palkovits punch method, it has been possible to demonstrate that aromatase activity is present in brain regions such as the preoptic area, the ventromedial, and the tuberal hypothalamus where estrogen receptors have been detected either by biochemical assays or autoradiography. However, this approach does not permit the determination of the exact anatomical localization of the enzyme and the receptor. Specifically, biochemical assays cannot evaluate to what extent aromatase and estrogen receptors are co-localized in the brain.

For a number of years, immunocytochemistry (ICC) has been used to visualize estrogen receptors in the brain (e.g., Greene, Sobel, King, and Jensen, 1984; in quail: Balthazart, Gahr, and Surlemont, 1989). In addition, we were recently able to identify by ICC aromatase-immunoreactive cells in the brain of the Japanese quail (Balthazart et al., 1990b and d).

The quail is a unique model to study the regulation of aromatase and the implication of this enzyme in the control of reproduction. The aromatase activity is high in quail compared to mammals (compare Schumacher and Balthazart, 1986 with

Roselli and Resko, 1984) and it changes dramatically (four- to sixfold increase) in response to T (Balthazart, Foidart, and Hendrick, 1990c; Balthazart, Schumacher, and Evrard, 1990g; Schumacher and Balthazart, 1986). The quail preoptic area contains a sexually dimorphic, T-sensitive nucleus. The medial preoptic nucleus (POM) is larger in males than in females (Viglietti-Panzica et al., 1986) and its volume increases following exposure to T (Panzica et al., 1987). The activation of copulatory behavior by T in quail critically depends on the action of T in the POM (Balthazart and Surlemont, 1990a). In addition, T must be aromatized in the POM in order to produce its behavioral effects (Balthazart, Evrard, and Surlemont, 1990a; Balthazart and Surlemont, 1990b). Finally radioenzyme assays on punched nuclei have shown that the preoptic aromatase activity in quail is essentially located in the POM (Schumacher and Balthazart, 1987; Balthazart et al., 1990g) and the recent immunocytochemical data have demonstrated that the aromatase-immunoreactive cells of the preoptic area are exclusively located within the POM and actually outline the borders of this nucleus (Balthazart et al., 1990b and d). Aromatase is therefore a specific neurochemical marker of POM and its presence in this nucleus is directly related to the control of male reproductive behavior.

The immunocytochemical studies have demonstrated that in quail, aromatase and estrogen receptors are usually found in the same brain areas (Balthazart et al., 1990d). This, however, does not prove that the two neurochemical markers are co-localized in the same cells. The two immunocytochemical procedures for aromatase and estrogens receptors have therefore been combined here to study the relative distribution of these markers in the quail brain. We demonstrate in this way that if a few cells contain both the enzyme and the receptors, in general, they are not co-localized in quail.

METHODS

Animals

Male Japanese quail (*Coturnix coturnix japonica*) were obtained from a local breeder at the age of 3–4 weeks. They were then kept in the laboratory for at least 3 weeks until they reached sexual maturity as confirmed by the presence of a large foam-producing cloacal gland. This structure is androgen-dependent (Sachs, 1967) and only develops when the testes of the birds begin to secrete testosterone at puberty (Ottinger and Brinkley,

1979). Throughout their life in the laboratory, all birds were exposed to a photoperiod of 16 h of light and 8 h of dark per day. They always had food (egg-laying hen flour) and water available *ad libitum*. A total of 13 birds were successfully stained for aromatase and/or estrogen receptors in the studies which are reported here.

The immunocytochemical localization of aromatase and of estrogen receptors was carried out either on fresh frozen or on perfused brains. In the first case, birds were killed by decapitation, their brain was rapidly dissected out of the skull, and frozen on powdered dry ice. Brains were stored in a deep freezer at -70°C for no more than 1 day until they were processed. When ICC was performed on perfused material, quail were deeply anesthetized with Hypnodil (Janssen Pharmaceutica, Belgium; 50 mg/kg body weight). They were then perfused with about 50 mL saline solution (0.15 M) followed by 200 mL of paraformaldehyde [4% in phosphate-buffered saline (PBS) 0.1 M, pH 7.2] or by 200 mL of Zamboni fixative (2% paraformaldehyde and 15% picric acid in 0.15 M phosphate buffer). Brains were then dissected and placed overnight in a 20% sucrose solution in 0.1 M PBS.

Immunocytochemistry

Single Label Immunocytochemistry. A standard peroxidase-antiperoxidase procedure using diaminobenzidine (DAB) as the chromogen was used for two birds in which aromatase and estrogen receptors were stained on alternate sections obtained from fresh frozen tissue. A minimum of 15 other birds had been used previously in this laboratory to establish in separate individuals the distribution of aromatase and estrogen receptors immunoreactivity (Balthazart et al., 1990b and d; Balthazart et al., 1989). The present study was performed to confirm the distribution of these markers in adjacent sections cut exactly in the same plane. Fresh frozen brains were cut on a cryostat in 25 μm transverse sections that were picked up on gelatin-coated slides. Two series of sections were alternatively collected. They were processed by ICC for aromatase and estrogen receptors, respectively. Sections were collected every 100 μm in the preoptic area (POA) starting at the level of the tractus septomesencephalicus and every 300 μm in the rest of the hypothalamus until the level of the oculomotor nerves.

Aromatase ICC was performed as described previously (Balthazart et al., 1990b). Sections were fixed for 30 min in 4% paraformaldehyde in PBS (phosphate buffer: 0.01 M, pH 7.2, NaCl: 0.125 M) at room temperature. Endogenous peroxidase was blocked by immersing the sections in a solution of 0.6% hydrogen peroxide in methanol for 20 min. Sections were incubated overnight at 4°C with the Harada's anti-aromatase antibody (Harada, 1988; see below) diluted at 1/1000 in PBS plus triton X-100 0.1% (PBST). On the next day, sections were processed according to the peroxidase-anti-

peroxidase (PAP) technique. The goat anti-rabbit serum (dilution 1/60 for 30 min) and PAP complex (1/300 for 30 min) were diluted in PBST. Extensive rinses in PBST were made between each step. The peroxidase was finally revealed by immersing slides for 6 min in a solution of DAB (20 mg in 50 mL PBST containing 20 μL of hydrogen peroxide at 30%). They were then dehydrated and mounted with Eukitt (O. Kindler, Freiburg, FRG).

For the estrogen receptor ICC, the procedure was similar in its principle and has also been previously described (Balthazart et al., 1989). Sections were fixed at room temperature with phosphate-buffered 4% paraformaldehyde, washed in PBS (pH 7.3, 0.025 M), and treated at room temperature with 0.1% Triton X-100 for 30 min. They were washed with PBS and then immersed for 30 min in 10% normal goat serum before incubation in the primary antibody H222SP γ (overnight, minimum concentration 0.1 $\mu\text{g}/\text{mL}$). After the incubation in the primary antibody, sections were rinsed and then incubated with the second antibody (goat-anti rat IgG, Abbott Diagnostic Products, minimum concentration: 10 $\mu\text{g}/\text{mL}$) and with the third antibody (rat peroxidase-antiperoxidase antibody, Abbott Diagnostic Products, minimum concentration: 0.5 $\mu\text{g}/\text{mL}$) for 30 min in each case. In order to enhance the detection of the receptors, we processed sections through the multiple-bridge technique (Blaustein and Turcotte, 1989b). They were incubated alternatively for three to five additional times in the second and third antibody before the binding was revealed by DAB. After each incubation with one antibody, sections were washed for 5 min in PBS. The binding was finally revealed by DAB and then sections were mounted as described above.

Double-Label Immunocytochemistry. The double label experiments were usually carried out on perfused brain ($n = 7$) because this provided a more precise localization of the antigens at the cellular level. After the overnight incubation in sucrose, brains were cut with a cryostat in 25 μm coronal sections, and they were then stained as free-floating sections. In addition, the same double-label procedure was also performed in four animals on fresh frozen sections mounted immediately after cutting on gelatin-coated slides to control for a possible decrease of immunoreactivity in the fixed material.

Estrogen receptors were first labeled by a technique identical to the one described above using always a multiple (3–5 $\times$) bridge technique to optimize the assay sensitivity. The binding of antibodies was revealed by DAB, which gave a brown precipitate. Aromatase was subsequently stained by a two-step procedure using a second antibody conjugated with alkaline phosphatase. It was then revealed by an incubation with a fast blue salt giving in the end a blue precipitate. This technique, which had been used before in single-label experiments (Balthazart, Foidart, Surlemont, Vockel, and Harada, 1990e), is briefly summarized below.

After staining the estrogen receptors, sections were

rinsed several times in a tris buffer-saline (TBS) (Tris buffer: 0.05 M, pH 7.6, NaCl: 0.15 M) and then incubated overnight with the primary anti-aromatase antibody diluted 1/1000 in TBST (TBS plus triton X-100: 0.5%). On the next day, they were incubated for 6 h in alkaline phosphatase-conjugated swine anti-rabbit immunoglobulins (Dakopatts D-306) diluted 1/20 in TBST. Extensive rinses in TBS were made between each step. Following incubations, the alkaline phosphatase was finally revealed for 15 min in a solution of fast blue (fast blue BB salt) (10 mg in 10 mL TBS 0.1 M, pH 8.2) containing 2 mg Naphtol AS-MS phosphate, 0.2 mL *N,N*-dimethylformamide and 0.01 mL Levamisole 1 M). Sections were mounted in an aqueous gelatin jelly as the blue chromogen is soluble in alcohol.

Antibodies and Controls

The anti-aromatase antiserum that was used is a polyclonal antibody raised in rabbit against human placental aromatase and purified by ammonium sulfate fractionation and affinity chromatography (Harada, 1988). This antibody appears to specifically recognize aromatase and to be monospecific as determined in classical biochemical (dose-dependent inhibition of aromatase activity in product-formation assays) and immunological (one precipitating line in an Ouchterlony double diffusion test, one immunoreactive band in Western blotting analysis) tests (see Harada, 1988 for more detail). It has also been demonstrated that this antibody completely inhibits aromatase activity in the quail brain without affecting the activity of other testosterone-metabolizing enzymes (Balthazart et al., 1990b). Additional controls including the omission of the primary, secondary, or tertiary antibody and the use of a preimmune rabbit serum in place of the primary antibody have all demonstrated that no residual signal is observed in these conditions. Finally, we have shown that when the primary anti-aromatase antibody is preabsorbed with an excess of purified human placental aromatase (see Harada, 1988 for the preparation and characteristics of this preparation), no immunoreactivity is observed in the quail brain.

We believe that, despite the fact that it was prepared with an antigen of human origin (human placenta), this antibody recognizes the only form of active aromatase in the quail brain. This conclusion cannot be based on biochemical characterizations since aromatase in the quail brain has never been purified but it relies on the properties of the antibody when tested on quail brain enzyme. The fact that aromatase activity can be *completely* suppressed by the addition of the antibody (Balthazart et al., 1990b) strongly suggests that it binds to all active enzyme molecules. In addition, we have shown that the distribution of aromatase activity as measured on punched nuclei matches closely the distribution of aromatase-immunoreactive cells: enzyme activity is found only in brain regions containing immunoreactivity and *vice versa* (compare

Balthazart et al., 1990b and d with Schumacher and Balthazart, 1987). Finally, the T-induced changes in aromatase activity in the preoptic area closely parallel the changes in immunoreactivity: after 5 days of exposure to T, there was a sixfold increase in enzyme activity, whereas the number of immunoreactive cells in the POM had been multiplied by five. All these data clearly support the notion that the present antibody recognizes the entirety of the active enzyme in the quail brain. Recent analysis by Western blotting suggests, in addition, that only one form of active enzyme is present in the quail brain since one single immunoreactive band was observed in the blots (J. Balthazart and C. DeRycker, unpublished results).

The monoclonal antibody made against estrogen receptors of human breast cancer tissue was provided by Abbott Diagnostic Products (Louvain-La-Neuve, Belgium). H222SP γ cross-reacts with estrogen receptors from a variety of tissues including mammalian uterus and brain (Greene et al., 1984; McClellan, West, Tacha, Greene, and Brenner, 1984; Blaustein and Turcotte, 1989b; Koch and Ehret, 1989), avian ovarian tissue (Greene et al., 1984), and the brain of songbirds (Gahr, Flügge, and Güttinger, 1987; Gahr and Konishi, 1988), and quail (Balthazart et al., 1989). It has been shown in song birds that cells accumulating tritiated estradiol in autoradiography are also labeled with the antibody H222SP γ (Gahr and Konishi, 1988). The staining of rat brains with H222SP γ is blocked by preadsorption with purified estrogen receptors (Cintra et al., 1986). We have previously demonstrated that in quail no residual staining with the present procedure is observed when the first, second, or third antibodies are omitted or the first antibody is replaced by normal rat immunoglobulins (Balthazart et al., 1989).

Additional controls for the specificity of these procedures were made in the double-label experiments. Some sections were incubated with the primary anti-aromatase antibody and then revealed by the PAP technique for estrogen receptors, while others were treated with the anti-estrogen receptors antibody and then revealed by the AP technique for aromatase. No staining was ever observed in these cases. Considering in addition that the techniques which were used for visualizing the two antigens use antibodies raised in different species (rat monoclonal and rabbit polyclonal) and enzymes (peroxidase and alkaline phosphatase) which are unlikely to present cross-reactions and that a sequential rather than simultaneous staining procedure was used, it appears that the two immunological procedures are specific. This statement is also supported by the observation that at the cellular level, the two antigens always displayed a different localization (cell nuclei for estrogen receptors and perikarya for aromatase), which was similar in the single- and in double-label procedures.

Data Analysis

The distributions of aromatase immunoreactive (ARO-ir) and of immunoreactive estrogen receptors (ER-ir)

cells in alternate serial sections through fresh frozen brains were first analyzed. All sections were examined under the microscope at final magnifications of 100–400 $\times$ and those showing immunoreactive cells were schematically drawn with the help of a camera lucida. The position of labeled areas were then plotted on semischematic drawings of the sections. A few selected areas showing both ARO-ir and ER-ir cells were then drawn in full detail with the camera lucida at a magnification of 100 $\times$. This analysis is summarized in Fig. 1.

The sections which had been stained by the double-label ICC were first screened for the presence of ARO-ir elements and their exact position in the brain was determined based on schematic drawings at low magnification (40 $\times$) made with the camera lucida (they had been

stained as free-floating sections, and their order therefore had to be reconstructed based on major neuroanatomical landmarks). In four birds (three perfused with 4% paraformaldehyde and one perfused with Zamboni fixative), the sections containing ARO-ir elements were then drawn at high magnification (100–400 $\times$), and the presence or absence of ER-immunoreactivity was noted in each ARO-ir perikaryon (Fig. 2). The sections that were quantified were selected to cover the different levels in the rostral-caudal axis of the three areas containing ARO-ir neurons (medial preoptic region, septum, and ventromedial to tuberal hypothalamus). In this analysis, ER-ir cells were divided in two groups (strongly or weakly labeled) depending on the intensity of the brown precipitate indicating the binding of the antibody against ER.

The neuroanatomical nomenclature used in this paper is based on previously published work on the quail and chicken brain (Baylé, Ramade, and Oliver, 1974; Kuenzel and Van Tienhoven, 1982; Kuenzel and Mason, 1988; Viglietti-Panzica et al., 1986).

RESULTS

Aromatase and Estrogen Receptors ICC on Alternate Sections

The localization of aromatase and estrogen receptors, which was observed by immunocytochemistry in alternate sections, confirmed the results of our previous studies (Balthazart et al., 1989, 1990b,d,e). These results are schematically presented in Figure 1.

Three groups of ARO-ir cells (large dots) are present in the quail brain. At the more rostral level, in the preoptic area (POA), the sexually dimorphic medial preoptic nucleus (POM) is specifically identified by this neurochemical marker (panels A–C). More than 50% of the ARO-ir cells of the quail brain are located in this nucleus. In the septal region, a group of labeled cells is found above the anterior commissure in the region ventral to the nucleus accumbens and, in a more caudal position, ventral to and also including the bed nucleus striae terminalis (panels D–F). At its most caudal extent, this cell cluster extends ventromedially to form a bridge toward the dorsal part of the nucleus paraventricularis magnocellularis (PVM). Finally, in the medial and caudal hypothalamus, an elongated cluster of immunoreactive cells runs from the dorso-lateral aspects of the ventromedial nucleus of the hypothalamus (VMN) to the nucleus inferioris hypothalami in the tuberal region (panels G–I).

On the alternate sections, immunoreactive es-

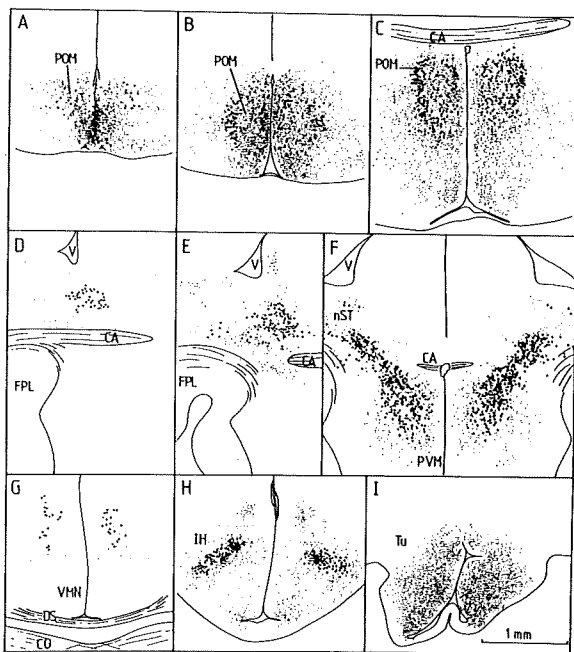


Figure 1 Camera lucida drawings of selected brain regions that contain aromatase immunoreactive neurons in the quail brain. In these drawings, each dot represents one immunoreactive cell. Large dots indicate aromatase, small dots are for estrogen receptors. Panels A–C represent different levels of the medial preoptic nucleus; panels D–F illustrate different aspects of the aromatase immunoreactive cells in the septal region; and panels G–I show different levels of the cluster of aromatase immunoreactive cells that runs from the nucleus ventromedialis hypothalami to the nucleus inferioris hypothalami in the tuberal region. In each series, panels are arranged in a rostral to caudal order. CA = commissura anterior; CO = chiasma opticum; DS = decussatio supraoptica; FPL = fasciculus prosencephali lateralis; IH = nucleus inferioris hypothalami; nST = nucleus striae terminalis; Tu = nucleus tuberis; V = ventriculus. Other abbreviations as in text.

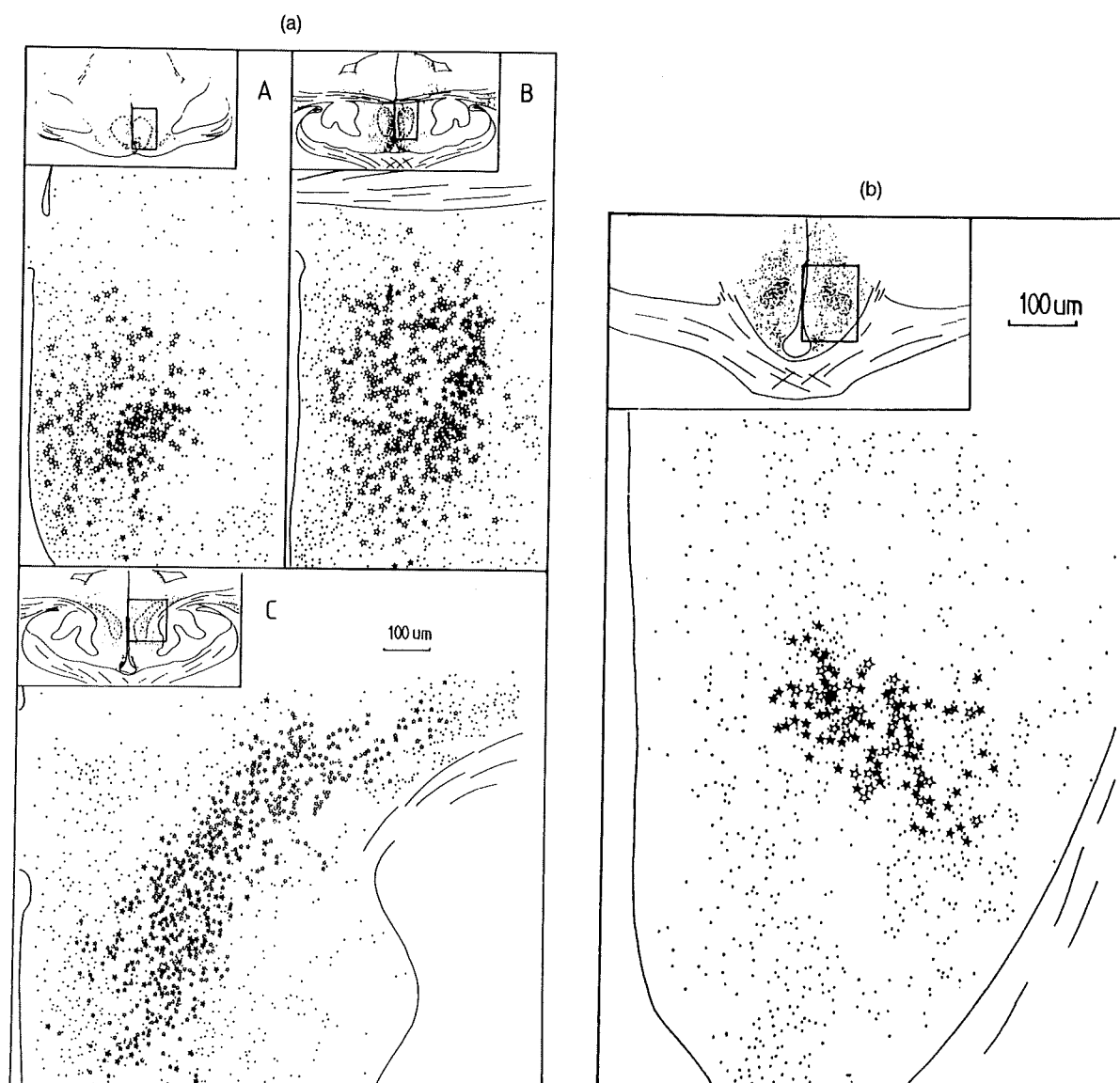


Figure 2 Camera lucida drawings of quail brain sections that had been double-labeled for aromatase and estrogen receptors. (a) Presents sections obtained at different levels of the POA in which only a small percentage of cell expressed at the same time aromatase and estrogen receptors. Panels: A = middle part of POM; B = POM at the level of the anterior commissure, C = caudal preoptic area and septum at the level of the nucleus striae terminalis. (b) Shows one section in the caudal hypothalamus at the level of the nucleus inferioris hypothalami. In each case, the insert indicates the location of the area that has been magnified. The rectangle defines the enlarged area and the dotted lines indicate the zone in which labeled cells were counted (see text for detail of results). The four panels in this figure correspond to panels B, C, F, and H in Figure 1. ● = cell labeled for estrogen receptor, ☆ = cell labeled for aromatase, ★ = cell double-labeled for ER and aromatase.

trogen receptors (small dots) were clearly present in all these areas that contained cells labeled for aromatase. The distribution of ER-ir was however broader. ER-ir cells were found in, but also around, the POM. They were also present in the septal region and in the ventromedial and tuberal

hypothalamus where they extended far beyond ARO-ir cells (e.g., panel I which is caudal to the last ARO-ir cells).

In most brain areas, ER-ir cells were by far more numerous than the ARO-ir ones. Cell counts were made in four sections corresponding to the

levels illustrated in panels B (middle part of POM), C (POM under the anterior commissure), F (level of the nucleus striae terminalis), and H (nucleus inferioris hypothalami) where ARO-ir cells are the most numerous. Even if counts of ER-ir cells were strictly limited to the area defined by the ARO-ir neurons, they were still in larger number with the exception of the region of the nucleus striae terminalis (level B: ARO-ir = 214, ER-ir = 1195; level C: ARO-ir = 400, ER-ir = 941; level F: ARO-ir = 583, ER-ir = 451; level H: ARO-ir = 157, ER-ir = 338). It must, however, be noted that in each case estrogen receptors were found outside the aromatase immunoreactive area and had these been included in the counts, they would have, by far, outnumbered the ARO-ir cells.

From these data, it is clear that ARO-ir cells are only found within regions also containing ER-ir elements, which confirms the close connection between these two neurochemical markers. This cannot however be taken as final evidence demonstrating their co-localization.

Double-Label Immunocytochemistry

The brain of 11 birds were processed through the sequential double-label immunocytochemistry for ER-ir and then ARO-ir cells. The colocalization of these two antigens was systematically quantified in the four birds that showed the darkest stainings. The pattern of results was however identical in the other birds. Clearly labeled ARO-ir cells were found in the same brain regions as in the single-label procedure. In this perfused material, the entire perikarya of the ARO-ir cells was stained and the blue precipitate extended in the cell processes far away from the cell nuclei (Fig. 3). Dense networks of immunoreactive fibers were clearly visible in the brain areas containing immunoreactive perikarya. As documented in Figure 3, many ARO-ir cells in the POM did not contain estrogen receptors.

Figure 2 contains exact representations of the distribution of ARO-ir, ER-ir and of double-labeled cells in a few selected brain areas as obtained in drawings with a camera lucida. Sections in the middle part of the POM, in the POM under the anterior commissure, in the preoptic area-septum at the level of the nucleus striae terminalis, and in the nucleus inferioris hypothalami are shown (levels similar to those presented in panels B, C, F, and H in Fig. 1). Visual inspection of this figure immediately reveals that most of the ARO-ir cells in the nucleus inferioris hypothalami contain at

the same time estrogen receptors [Fig. 2(b)]. The situation is quite different in the preoptic area where the percentage of co-localization between the two neurochemical markers is quite low [Fig. 2(a), panels A, B, C]. The situation shown in this figure is representative of the entire population of birds that was studied: the animal drawn in this figure was actually selected because it displayed a percentage of co-localization close to the mean of all data (see below). In these sections like in those from other birds, the double-labeled cells were randomly distributed within the population of ARO-ir neurons. No preferential localization could be detected.

The quantitative evaluation of the co-localization of the two markers in the brain of four birds is summarized in Table 1.

Only about 15% of the ARO-ir cells in the POM were at the same time immunoreactive for estrogen receptors. This included a small percentage of cells in which the brown DAB precipitate indicating the presence of ER was extremely light. The co-localization of ARO and ER was even lower in the septum where <5% of the ARO-ir cells were double-labeled even when weakly ER-labeled cells were included. By contrast, a completely different situation was seen in the ventromedial and tuberal region of the hypothalamus. In the cellular group of ARO-ir neurons extending from the VMN to the nucleus inferioris hypothalami, >60% of ARO-ir elements were double-labeled. This high proportion of cells in which both antigens were present was observed from the rostral to the caudal part of this cell group. We could not detect subregions in which a systematically higher or lower co-localization would be present. The same conclusion was reached for the POM and the septal region, which showed fairly stable levels of co-localization from the rostral to the caudal sections. The use of Zamboni instead of paraformaldehyde as fixative did not seem to influence markedly the results (data for the Zamboni-perfused animal are within the range of the other birds).

In each double-labeled section that was examined, both ER-ir and ARO-ir cells were found which excluded the possibility of false-negative results. Furthermore, in almost each section that was counted (25 out of 26 in POM, 16 out of 20 in the septum, and 31 out of 31 in the hypothalamus), a few double-labeled cells at least were found demonstrating that the absence of double-label in the vast majority of the cells was not likely to be caused by some artifactual interaction between the two procedures.

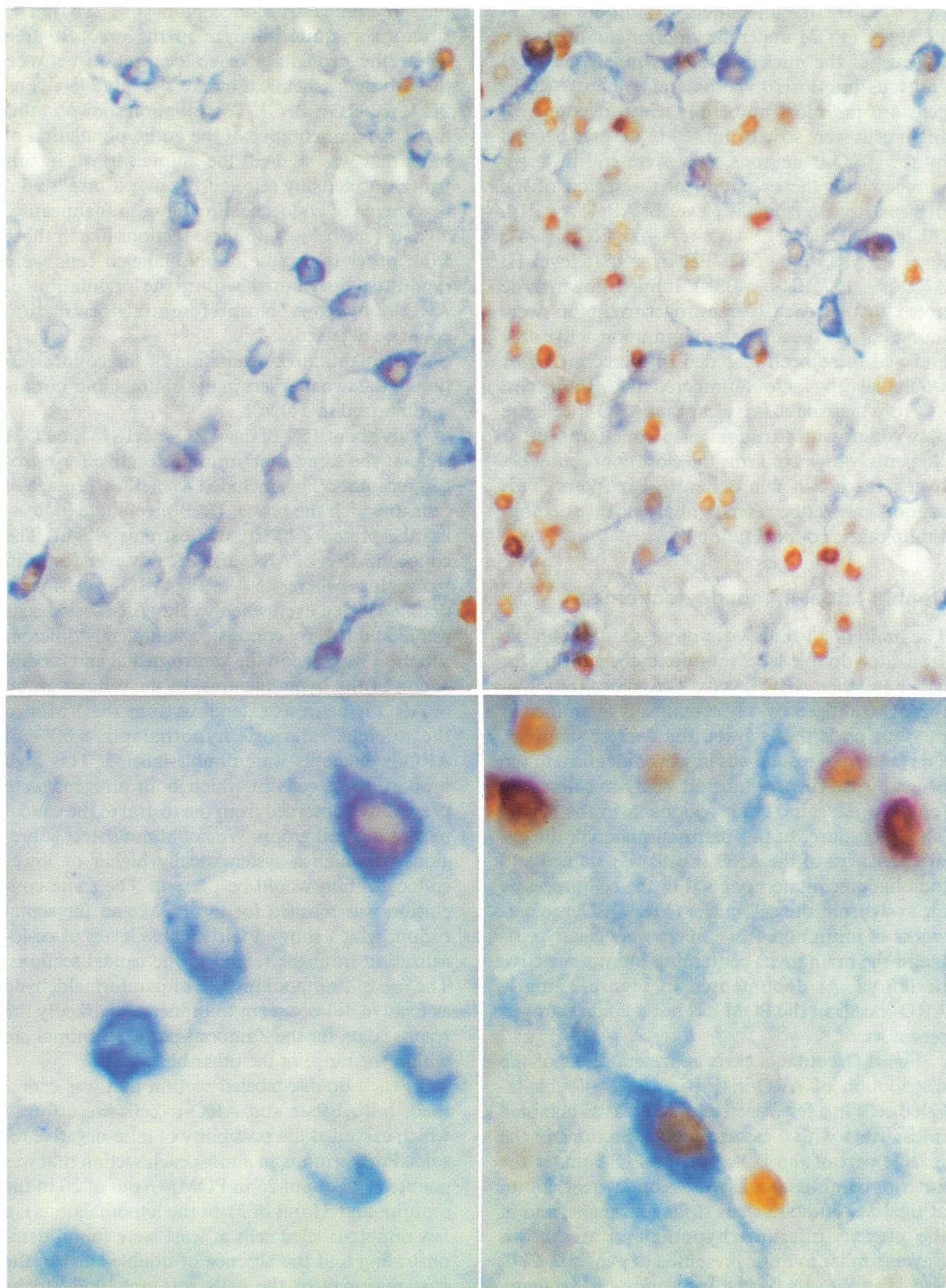


Table 1 Quantitative Analysis of the Co-Localization of Aromatase and Estrogen Receptors in the Quail Brain

	POA (<i>n</i> = 4)	Septum (<i>n</i> = 4)	Hypothalamus (<i>n</i> = 3)
Number of sections counted/animal (range)	5–8	4–6	8–13
Number of ARO-ir cells counted/animal	1516 ± 228	399 ± 75	616 ± 33
% with strong ER-ir (range)	14.37 ± 5.27 (5.15–27.37)	2.17 ± 0.72 (0.72–3.59)	58.99 ± 15.16 (28.66–74.28)
% with weak ER-ir (range)	2.21 ± 0.37 (1.33–3.04)	1.68 ± 0.99 (0.00–4.58)	8.84 ± 6.11 (1.80–21.04)
% double-labeled cells (total) (range)	16.58 ± 5.00 (8.22–29.29)	3.86 ± 1.59 (1.15–8.17)	67.71 ± 9.01 (49.70–77.98)

Counts were made separately in three regions of the brain: the preoptic medial nucleus of the preoptic area (POA), the septal region ventral to the nucleus accumbens, and the bed nucleus of the striae terminalis (Septum) and the cluster of cells running from the dorsal part of the VMN to the nucleus inferioris hypothalami (Hypothalamus). Data are means ± standard errors (range in parenthesis) of four males perfused with 4% paraformaldehyde (*n* = 3) or Zamboni fixative (*n* = 1).

We also evaluated in a few sections (those represented in Fig. 2) the total number of ER-ir cells that were located within the area containing aromatase immunoreactivity (region delimited by the dotted line in the inserts). The relative number of ER-ir and ARO-ir cells (E/A) estimated in this way in the double-labeled sections was very similar to the corresponding ratio calculated from the counts obtained in the adjacent sections (see detail of counts above in the section on alternate sections) labeled for one or the other antigen (middle level of POM: E/A = 1104/294 = 3.75 compared to 5.58 in adjacent sections; POM under commissure: E/A = 1122/594 = 1.88 compared to 2.35 in adjacent sections; region of the nucleus striae terminalis: E/A = 930/1020 = 0.91 compared to 0.77 in adjacent sections and nucleus inferioris hypothalami: E/A = 560/220 = 2.54 compared to 2.15 in adjacent sections). The differences observed between the single- and double-label immunocytochemistry in the relative numbers of cells labeled for one or the other marker are well

within the range of the inter- or even intra-individual (from one section to the other) differences. This again supports the notion that the two immunocytochemical procedures used on the same sections did not interfere with the identification of either ARO-ir or ER-ir cells. Based on these quantitative data, it seems very unlikely that such a technical artifact might explain why >80% or ARO-ir cells in POM do not appear to contain estrogen receptors.

Systematic counting of the ER-ir neurons was not performed since the distribution of these cells is much wider than for aromatase and the comparison of their number with the number of ARO-ir and of double-labeled cells would be totally artificial and determined only by the limits of the area selected for quantification. In the zone containing ARO-ir cells in the four sections drawn in Fig. 2 (area delimited by the dotted line in the inserts), the counts described above suggested, however, that only a small fraction of the cells containing estrogen receptors are at the same time aromatase

Figure 3 Microphotographs demonstrating aromatase immunoreactive and immunoreactive estrogen receptor cells in the quail preoptic area. The two pictures at the top are low magnifications in the part of the medial preoptic nucleus that contains double-labeled cells. The two bottom pictures are high magnifications of the same region illustrating one double-labeled cell with weak estrogen receptor-immunoreactivity (left) and one double-labeled cell with strong estrogen receptor-immunoreactivity (right), as well as several cells that are only labeled for aromatase (blue) or for estrogen receptors (brown). The presence of aromatase-immunoreactive cellular processes is very noticeable in the low magnifications. Please note that the photographed areas were selected to illustrate the co-localization of both antigens in the same cells but they are not representative of the relative frequency of this co-localization in the POA.

immunoreactive (middle level of POM: 8.15%, POM under commissure: 16.04%, level of the nucleus striae terminalis: 17.63%, nucleus inferioris hypothalami: 30.18%). This result is in full agreement with our previous estimations of the relative numbers of ER-ir and ARO-ir cells in the POM. We evaluated indeed that >90% of the cells in POM contain estrogen receptors (Balthazart et al., 1989) while only 10–15% are labeled for aromatase (Balthazart et al., 1990d). It is therefore obvious that only a small fraction of ER-ir cells also contain aromatase.

DISCUSSION

By combining immunocytochemical procedures that had been used before to label aromatase and estrogen receptors in the quail brain, we were able to study here the anatomical relations between these two neurochemical markers. Unexpectedly, we have demonstrated in this way that a large proportion of the aromatase immunoreactive cells in the quail brain do not contain immunoreactive estrogen receptors. The specificity of the immunocytochemical procedures for both ER and ARO has been established previously (Balthazart et al., 1989, 1990b) and need not be discussed here again. Biochemical, immunological, and physiological arguments concur to demonstrate that the immunocytochemical signals are specific.

The use of double-labels in immunocytochemistry, however, raises additional problems. False-positive signals might be observed due to cross-reactions between the two procedures. This possibility was minimized here by the use of primary antibodies raised in different species (rat monoclonal for ER and rabbit polyclonal for ARO) and of different enzyme markers in the two systems (peroxidase and alkaline phosphatase) as well as by the choice of a sequential procedure for the staining. We showed in addition that the goat anti-rat secondary antibody did not interact with the rabbit polyclonal antibody to aromatase and that the swine anti-rabbit secondary antibody did not interact with the rat monoclonal antibody to ER. We had also shown previously that the ER-immunoreactivity was exclusively nuclear while the aromatase immunoreactivity was only detected in the perikarya always leaving a clear cell nucleus. The same cellular distribution of the two antigens was observed in the double-label procedure, which also confirmed its specificity.

As one major finding of this study was the ab-

sence of co-localization of ER and aromatase, the possibility of a false-negative result (for ER) was of major concern here. To minimize this risk, we always stained ER before the aromatase by a procedure identical to the one that had been used and validated previously. In addition, the sensitivity of the ER detection was increased by the use of the multiple-bridge technique, which resulted in very intense staining for this antigen. The nature of the results also allow us to exclude an artifactual absence of co-localization. In each section where ARO-ir cells not containing ER were observed, there was always a number of cells intensely stained for ER demonstrating that these had been successfully labeled in the particular section. In addition, most of these sections also contained a few double-labeled cells indicating that there was no negative interaction between the two immunocytochemical procedures at a cellular level. Furthermore, the relative numbers of ARO-ir and ER-ir cells labelled in adjacent (single-label) or on the same (double-label) sections were very similar. All these observations demonstrate that there was no interaction between the two immunocytochemical procedures and that the relative distribution of ER and aromatase immunoreactivity seen by this method accurately reflects the distribution of the immunoreactive antigens in the brain.

The absence of detectable ER in the ARO-ir cells might also result from an artifact that would be of physiological rather than strictly technical nature. The monoclonal antibody H222SP γ recognizes one epitope that is located in the hormone-binding domain of the ER molecule (Kumar, Green, Staub, and Chambon, 1986). It has therefore been suggested that the free and occupied receptors might be differentially recognized by H222SP γ . Based on experimental studies, claims have been made that this antibody recognizes equally well both forms of receptors (Press and Greene, 1987) or recognizes preferentially if not exclusively the free receptor (Blaustein and Turcotte, 1989a; Reger, Gerall, and Givon, 1989). In one recent study, the cytoplasmic immunostaining, which had been obtained by a multiple-bridge technique on brains of ovariectomized guinea pigs, was almost completely lost following the injection of estradiol (Blaustein and Turcotte, 1989a). However, the loss of nuclear immunostaining was only minimal. In addition, biochemical studies have shown that H222SP γ binds to both occupied and free ER even if the molar ratios for these interactions are different (Giambiagi and Pasqualini, 1990). We believe therefore that the

high level of receptor occupancy which might be present in ARO-ir cells is not sufficient to explain the absence of visualizable receptor in these cells.

This statement is further supported by our experimental results. We used a multiple-bridge technique providing high sensitivity to stain ER. Intense staining was obtained as a consequence as reflected by the observation that in each brain area studied, the vast majority of the positive cells were darkly stained (compare number of strongly and weakly stained cells in Table 1). This suggests that the sensitivity of the immunocytochemical method was adequate (few cells were at the detection limit) and that we did not ignore a large population of neurons that were beyond the limits of sensitivity of the technique. Furthermore, in a recent study we found no difference in the number of ER-ir cells in the brain of male and female quail (Balthazart et al., 1989). As it can be expected that the level of receptor occupancy is quite different as a function of the sex, this means that if receptor occupancy modulates the intensity of the immunoreactivity it probably does not drastically change the number of visualizable immunoreactive cells.

Independent of the problems related to the detection of the antigens, their real concentration might be modulated by the hormonal milieu, and since these experiments were performed on intact birds, the absence of co-localization could in theory result from a down-regulation of one or the other antigen in cells that potentially express them in adequate conditions. This interpretation also appears unlikely. We showed previously that T dramatically increases the number of ARO-ir cells in the preoptic area. The birds used here were sexually mature and were exposed to high-plasma T level. They were therefore in optimal conditions for the detection of cells expressing aromatase as reflected by the large number of ARO-ir cells found in their brain (Table 1). On another hand, Schlinger and Callard (1989b) have demonstrated by binding assays that the total concentration (free plus occupied) of estrogen receptors in the quail hypothalamus is increased by T. Again as birds here were sexually mature, they were in optimal conditions as far as estrogen receptor expression is concerned. Given that the concentration of these receptors is doubled in T-treated birds by comparison with castrates (Schlinger and Callard, 1989b), they should have been identified in most of the cells of intact birds which express them. The possible decrease in immunoreactivity of occupied receptors (one molecule of occupied estrogen recep-

tor only binds one molecule of H22SP γ while a molecule of free receptor binds two; Giambiagi and Pasqualini, 1990) should in theory be totally compensated by the increased concentration of antigen.

The fact that approximately 85% of the ARO-ir cells in the POM do not contain ER raises important questions concerning the regulations of aromatase activity and physiological role of the estrogens produced centrally. It is known that testosterone increases the preoptic aromatase activity in many species (see Introduction). In rats, this effect appears to be mediated, at least in part, by the interaction of the steroid with androgen receptors as the increase in enzyme activity can be prevented by a treatment with the antiandrogen, flutamide (Roselli and Resko, 1984). However, it has also been shown in several independent studies on quail (Schumacher et al., 1987), ring dove (Hutchison and Steimer, 1986), and rat (Steimer et al., 1989) (but see Roselli and Resko, 1984 for contradictory evidence) that a treatment of castrated males with estrogens (estradiol benzoate or diethylstilbestrol) increases aromatase activity in the preoptic area-anterior hypothalamus. This suggested a feedback effect of the product of enzyme on the activity of the enzyme itself. The most parsimonious interpretation of the induction by estrogens of aromatase activity was that estrogens formed in one neuron by aromatization or coming from the periphery (e.g., testicular production) interacted in the cell nucleus of ARO-ir cells with ER to induce the synthesis of new enzyme molecules. If 85% of the ARO-ir cells lack ER, an alternative model needs obviously to be proposed. It is of course possible that the aromatase induction, which had been seen following treatment with fairly high doses of estradiol, resulted from an interaction of this steroid with androgen receptors. Estradiol is indeed able to significantly displace androgens from their receptors (Sheridan, 1983; Harding, Walters, and Parsons, 1984; Bonneau, Ahdieh, Thornton, and Feder, 1987) and at high concentrations, this effect might become biologically relevant. This interpretation cannot however take into account the effects of diethylstilbestrol on aromatase activity (Schumacher et al., 1987; Hutchison and Steimer, 1986) as this compound does not bind to the androgen receptor (Chamness, King, and Sheridan, 1979; Sheridan, 1983). The possibility that estrogens regulate aromatase activity through a transsynaptic mechanism needs therefore to be contemplated. It has been shown that the noradrenergic agonists are able to regulate

aromatase activity in the pineal gland (Cardinali, Ritta, and Gejman, 1982) or in the testes (Verhoeven, 1980), and similar mechanisms might take place in the hypothalamus (Canick et al., 1987; Raum and Swerdloff, 1981; Raum, Marcano, and Swerdloff, 1984). As the noradrenergic activity in the brain appears to be under the control of estrogens (McEwen and Krey, 1984), this estrogen-sensitive noradrenergic transmission might represent the neuroanatomical substrate underlying the regulation of aromatase activity by estrogen. It is important to mention that, in quail, the brain areas demonstrating high densities of ARO-ir neurons (medial preoptic nucleus and tuberal hypothalamus) contain very high concentrations of norepinephrine as determined by high-pressure liquid chromatography (HPLC) (Balthazart, Libiouille, and Sante, 1988a; Balthazart and Ball, 1989) and high densities of α_2 adrenergic receptors identified by quantitative autoradiography (Ball, Nock, McEwen, and Balthazart, 1989; Balthazart and Ball, 1989). The fact that, in quail, a denervation of the brain by the specific noradrenergic neurotoxin DSP4 enhances aromatase activity in the preoptic area (Balthazart and Ball, 1989) certainly supports the present interpretation. Further studies should now analyze the precise anatomical relationships between noradrenergic cells and projections, estrogen receptors and aromatase.

The absence of estrogen receptors in the aromatase immunoreactive cells also raises questions concerning the behavioral effects of estrogens formed by central aromatization. T activates male sexual behavior by acting specifically in the sexually dimorphic medial preoptic nucleus as demonstrated by stereotaxic implantation and electrolytic lesions (Balthazart, Surlemont, Foidart, and Schumacher, 1988b; Balthazart and Surlemont, 1990a). It is known that the activation by T of copulatory behavior in quail requires the aromatization of the steroid in the preoptic area and aromatase inhibitors given either systemically or directly implanted in the POM suppress or strongly delay the restoration by T of the behavior in castrates (Balthazart et al., 1990a, and c; Balthazart and Surlemont, 1990b). Aromatase activity and aromatase immunoreactive neurons are localized specifically in the POM (Schumacher and Balthazart, 1987; Balthazart et al., 1990b,d, and g). The estrogens produced by central aromatization must interact with estrogen receptors to activate the behavior because antiestrogens, such as tamoxifen or CI-628, given systemically or directly implanted in

the POM are able to completely block the behavioral effects of T (Alexandre and Balthazart, 1986; Balthazart and Surlemont, 1990b). It was therefore expected that estrogen receptors would be co-localized with aromatase, at least in the POM, where the biological role of the estradiol derived from T-aromatization is so well established. The fact that this co-localization was observed in only 15% of the aromatase-immunoreactive cells is very surprising because it is unlikely that estrogens produced by central aromatization of T exert their biological effects by acting on other ER containing cells after a passive diffusion through the inter-cellular space.

The limited co-localization observed here suggests the existence of two types of ARO-ir neurons. A few of them contain both aromatase and estrogen receptors. The estrogens produced in these cells might act, at least in part, through the classical genomic mechanisms resulting in the production of new proteins after the interaction of occupied ER with specific portions of the DNA. In the other ARO-ir cells, no ER are present and estrogens would act by some other unconventional mechanism. The same type of nongenomic action might, of course, also take place in cells containing estrogen receptors. It is important to note here that, in quail and in rat, biochemical studies have demonstrated the presence of high levels of aromatase activity in synaptosomes prepared by differential centrifugation (Steimer, 1988; Schlenger and Callard, 1989a). In addition, we have recently shown by preembedding immunocytochemistry and electron microscopy that numerous synaptic boutons containing aromatase immunoreactive material are present in the quail preoptic area (Naftolin, Leranthe, and Balthazart, 1990b; Balthazart et al., 1990f). Based on this evidence, it appears likely that significant amounts of estrogens are produced at the synaptic level. They might produce non ER-mediated actions. These might include previously described synaptogenic/synaptolytic effects of estradiol (Naftolin et al., 1990a; Naftolin and Brawer, 1978). Estrogens also appear responsible for the fluctuations of the post-synaptic membrane organization during the ovarian cycle (Naftolin et al., 1990a). Their synthesis at the synapse level is therefore biologically very relevant. On another hand, estrogens and their metabolites, the catecholestrogens are potent catechol *O*-methyl transferase inhibitors and in general modulate the synthesis, metabolism, and binding of catecholamines (MacLusky et al., 1984; McEwen and Krey, 1984). Their presence in the

synaptic zone might directly regulate the activity of neurotransmitters.

The demonstration of two types of aromatase-immunoreactive cells in the brain therefore raises a number of new questions concerning the biological role of central aromatase and the mode of action of the estrogens produced by this enzymatic process. Whether both types of ARO-ir cells (containing or not ER) are implicated in behavior activation remains unclear. The fact that antiestrogens, such as nitromifene citrate or tamoxifen, block the activation of behavior by T (Alexandre and Balthazart, 1986; Balthazart and Surlemont, 1990b) suggests that the interaction with classical ER is critical but does not rule out the possibility that direct effects of estrogen at the synapse level might be important. The biological role of the aromatase present in the septum is not elucidated at present but, based on the fact that virtually none of the aromatase immunoreactive cells contain at the same time ER, it seems likely that estrogens produced at this level act through mechanisms that are not mediated by the classical nuclear ER. By contrast, classical actions of estrogens through binding with nuclear receptors might be predominant in the hypothalamus. Additional studies should now be undertaken to elucidate the respective role of these two types of aromatase-containing cells.

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