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Immunocytochemical Localization of Androgen Receptors in Brains of Developing and Adult Male Rhesus Monkeys

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We localized immunoreactive androgen receptors in the central nervous system (CNS) of fetal and adult male rhesus macaques by immunocytochemisty using an affinity-purified polyclonal antibody to the first 21 amino acids of the human androgen receptor (AR). This antibody caused a shift in the mobility of AR-bound ^{3}H-DHT on a sucrose gradient and recognized a protein of approx 116 kDa on Western blot. Other criteria for specificity are presented. We localized AR in the diencephalon of male rhesus monkey fetuses. Immunoreactive neurons were found in the medial hypothalamic area and the ventromedial nucleus of the hypothalamus on days 47, 61, and 124 of gestation. At 124 d of gestation, AR immunoreactivity was also found in the arcuate nucleus. AR immunostaining was not found in other diencephalic structures in fetal life, including the preoptic area. In the adult monkey, neurons in ventromedial, dorsomedial, and arcuate nuclei of the hypothalamus; cortical, medial, and accessory basal nuclei of the amygdala; and regions of the hippocampus and the anterior pituitary gland contained immunoreactive AR. These data indicate that AR is found in specific areas of the CNS early in fetal development, but they also appear in other brain areas as the fetus grows. At 124 d of gestation (term, 167 d), the hypothalamic location of immunoreactive AR is similar to the adult.

Key Words: Androgen receptor; brain; fetus; primate; immunocytochemistry.

Introduction

Circulating androgens produce a wide spectrum of biological effects in fetal and adult primates *(1,2)*. In the fetus, prenatal androgen organizes brain areas that mediate reproductive behaviors in infancy and adulthood. In the adult, androgens regulate gonadotrophin secretion *(3)*, reproductive *(4)* and aggressive behavior *(5)*, and growth and secretion of accessory organs of reproduction *(6)*. Circulating androgens act on specific target tissues (such as the brain, pituitary, prostate, and seminal vesicle), through an intracellular androgen receptor (AR). The AR appears to reside primarily in the cell nucleus, where the ligand–AR complex binds to specific regions of DNA, affecting gene transcription, including autoregulation of AR mRNA *(7,8)*.

The distribution of AR immunoreactivity in peripheral reproductive tissues has been well characterized. In the rat prostate *(7,9,10)* and rhesus monkey seminal vesicle *(11)*, AR immunostaining is localized in nuclei of ductal epithelial and periacinar stromal cells. In human and rat testes, nuclei are stained in the peritubular, Leydig, and Sertoli cells *(12,13)*, as well as the arterial musculature *(13)*. AR immunoreactivity has also been demonstrated in the efferent ductules and the epididymis of rhesus monkeys *(14)*. Immunostaining has been used to localize AR within the central nervous system (CNS) of rats, hamsters, guinea pigs, and two species of primate (*Macaca fasicularis* and *Macaca mulatta*) *(15–19)*. In the guinea pig, staining is localized to the nucleus *(18)*, whereas in the rat, hamster, and primate, cytoplasmic staining in addition to nuclear staining was found *(17,19,20)*.

In this article, we describe the distribution of AR in target tissues of rhesus monkeys (*M. mulatta*) using an affinity-purified antibody directed to the N-terminus of the human AR (aa 1–21). Antibodies made from this same peptide have been shown to be specific for the AR in rats *(10)* and provide a promising tool for probing potential changes of AR in target tissues during different physiological states *(19–21)*. We used this antibody to study the distribution of AR immunoreactivity in androgen-dependent

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target tissues of the adult male rhesus monkey for the purpose of validating its use in this species. Thereafter, we compared the ontogeny of AR in the brain of the nonhuman primate fetus and compared the results with data obtained from three adult rhesus monkeys and a limited amount of published data from other laboratories on tissues from nonhuman primates.

Results

Validation of Immunocytochemistry (ICC)

We used rhesus monkey myometrial cytosol as a source of primate AR. The addition of anti-AR antibody to cytosol produced a shift in the migration of ^{3}H-DHT-bound AR in a sucrose gradient, which did not occur when irrelevant antibody or no antibody was added (Fig. 1). In the presence of anti-AR antibody, the ^{3}H-DHT-bound receptor migrated lower (i.e., higher concentration of sucrose) on the gradient, indicating that our antibody binds to AR. Concurrent with the sucrose gradient assay, we performed a receptor binding assay on aliquots of the myometrial cytosol. Cytosolic AR-bound 27.6 fmol ^{3}H-DHT/mg soluble myometrial protein.

Western immunoblotting was performed to characterize further the specificity of this anti-AR antibody. Immunoreactive bands were seen at 117, 65, and 42 kDa. The largest band corresponds to the molecular weight of human AR, whereas the smaller bands are thought to be proteolytic fragments *(10)*.

The prostate and seminal vesicle are androgen-dependent accessory organs of reproduction. We found strong AR immunoreactivity in nuclei of ductal epithelial cells, and in nuclei of periacinar smooth muscle cells of both the prostate and seminal vesicle (Fig. 2A,B). Adjacent sections incubated with preimmune antibody (Fig. 2C,D) or immune antibody preadsorbed with the antigenic peptide (Fig. 2E,F) did not show AR immunoreactivity. These observations served as validation of our ICC method for localizing AR, because they are similar to the staining patterns found by others using human prostate *(21)* and monkey seminal vesicle *(11)*.

AR immunoreactivity in the CNS was found in areas that also show AR by binding assays on brain tissues of nonhuman primates *(22,23)*. Specific immunoreactivity was localized in neuronal nuclei of all brain areas analyzed. In the diencephalon, strong AR immunostaining was found in the ventromedial (VMN) and dorsomedial (DMN) hypothalamic nuclei (Fig. 3A–D). Staining density was consistent throughout the length of these nuclei. The VMN becomes smaller and slightly more lateral in its most caudal region. AR immunostaining follows this anatomic distribution. Likewise, specific staining of the DMN disappears at its caudal boundary.

Moderate immunoreactive staining was found in the lateral hypothalamic area and in neurons of the arcuate

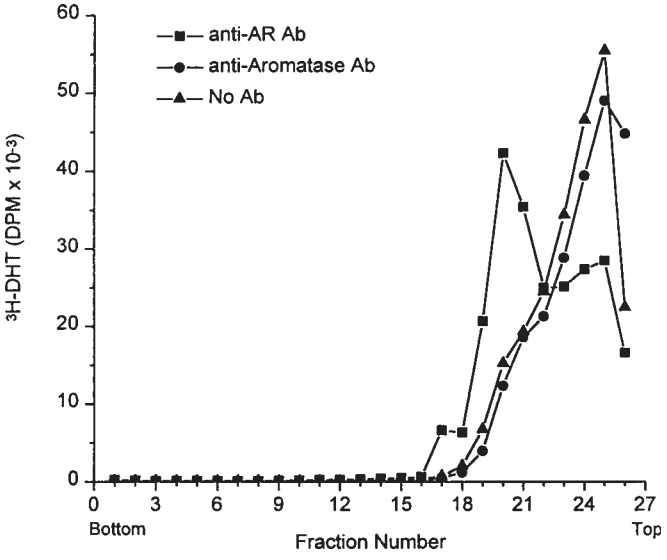

Fig. 1. Myometrium cytosols were incubated with 3 m*M* ^{3}H-DHT and then with either anti-AR antibody or controls (antiaromatase antibody or no antibody). Each incubate was loaded on 5–20% linear sucrose gradients and centrifuged at 205, 600*g*. Binding of the AR-specific antibody produced a shift of the peak ^{3}H- DHT-bound moiety from Fraction 25 (control antibody) to Fraction 20 (AR-specific antibody).

nucleus, which begins near the caudal border of the DMN (Fig. 4A,B). AR immunostaining was also observed in limbic structures of the temporal lobe. AR immunoreactive nuclei are widely distributed in the CA1-CA3 regions as well as the dentate gyrus of the hippocampus (Fig. 4C,D). The anterior pituitary gland contained AR immunoreactivity in nuclei of pituicytes (Fig. 4E), but no staining was seen in adjacent sections incubated with preimmune serum (Fig. 4F).

Dark staining was found in the cortical amygdaloid nucleus, with moderate staining in the medial and basal amygdaloid nuclei (Fig. 5A–C). As with the diencephalic nuclei, immunostained cells are seen throughout the rostral-caudal extent of the amygdaloid nuclei. Adjacent sections incubated with antigen peptide-preadsorbed primary antibody did not show AR-immunostained cells (Fig. 5D). The observations presented above were consistent among the tissues of the three animals that were studied.

AR Localization in Fetal Brains

Following the validation of the ICC in adult tissues, we localized AR-immunoreactive neurons in the diencephalon of fetal male rhesus monkeys. Immunostaining was concentrated in specific brain areas. In the 47-d fetus, we found immunostained neurons in the medial hypothalamic area and in the VMN (Fig. 6). Likewise, in the d 61 fetal brain, we found AR immunoreactivity in the medial hypothalamic area and in the VMN (Fig. 7). In the diencephalon of the 124-d fetus, AR immunostaining was found in the medial hypothalamic area, the VMN, and the ARC (Fig. 8). In general, distribution of AR immunostaining was consis-

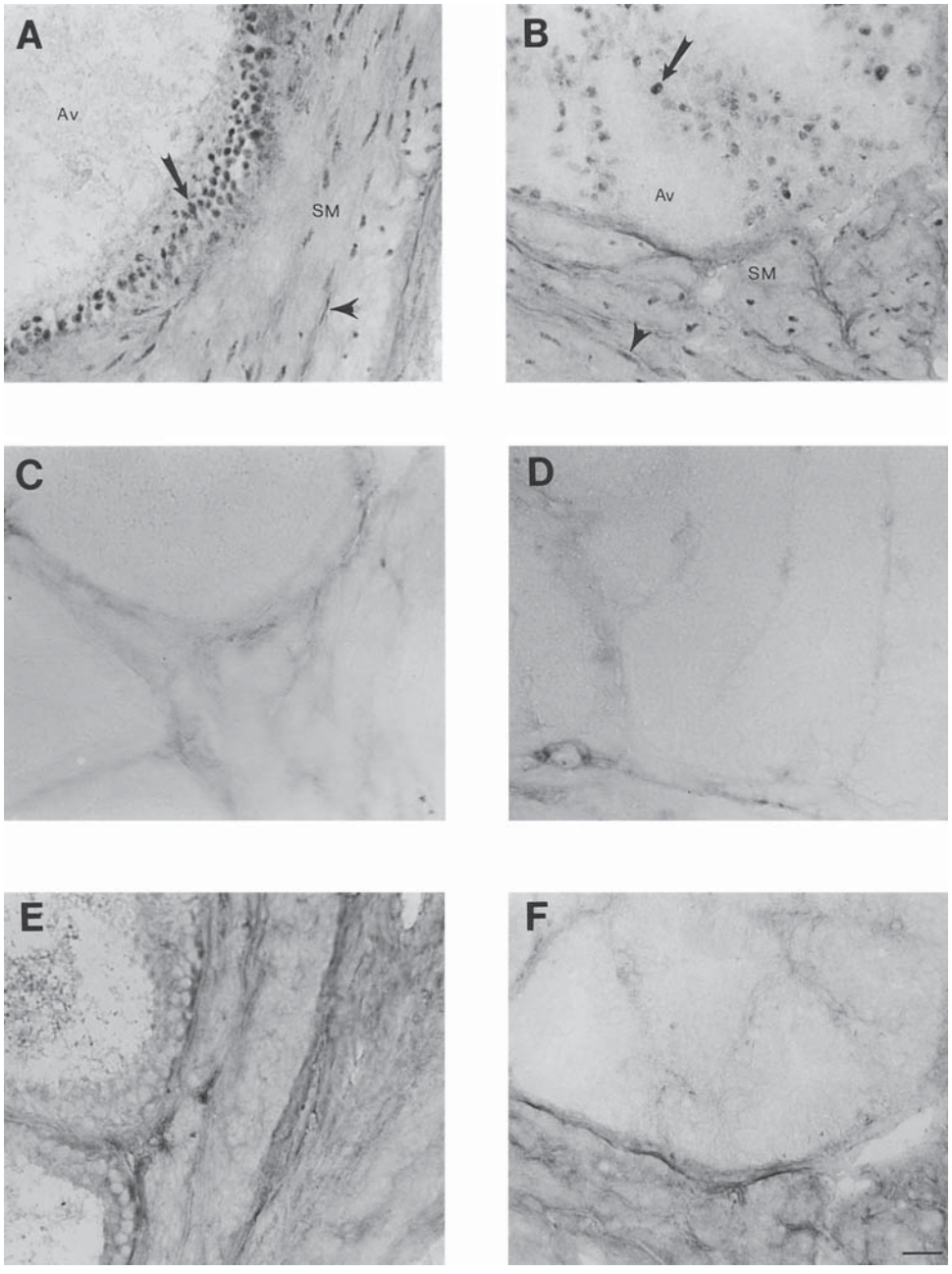

Fig. 2. Prostate and seminal vesicle from an adult male rhesus macaque were stained for AR immunoreactivity. AR-specific immunostaining was found in nuclei of ductal epithelial cells (arrow) and periacinar smooth muscle cells (arrowhead) of the prostate **(A)** and seminal vesicle **(B)**. Sections of the prostate **(C)** or seminal vesicle **(D)** incubated with preimmune antibody showed no staining. Likewise, sections incubated with anti-AR antibody preadsorbed with the antigenic peptide showed no staining in the prostate **(E)** or seminal vesicle **(F)**. Av, alveolus; SM, smooth muscle. Bar = 20 μm.

tent among the three gestational ages, with the exception of the ARC. AR-positive neurons appear caudal to the preoptic area and are seen throughout the medial hypothalamic area, including cells in the VMN. As in the medial hypothalamic area, AR immunoreactivity is found throughout the rostral-caudal region of the VMN. At 124 d of gestation, AR immunostaining of neurons in the ARC is evident medial and ventral to the VMN. AR-immunoreactive neurons were not found in other diencephalic nuclei, including the preoptic area.

Discussion

We have localized immunoreactive AR in the diencephalon of adult and fetal rhesus monkeys using antibodies that recognize nuclear AR. We have shown AR immunostaining in the medial hypothalamic area and VMN of early and midgestation fetuses. In addition, AR immunoreactivity was found in the ARC on d 124 of gestation. Circulating androgens can be measured as early as d 38 of gestation in male monkey fetuses *(24)*. Levels of circulating T are highest early in gestation and then decrease during the later

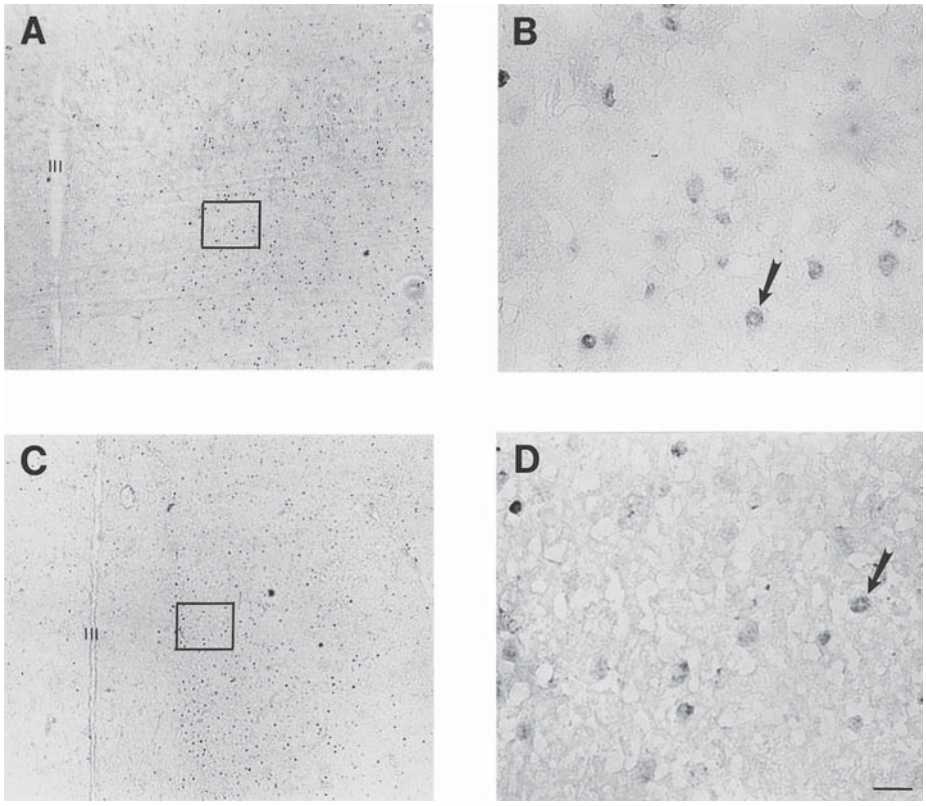

Fig. 3. Sections from the diencephalon of an adult male rhesus monkey were stained with anti-AR antibody. Immunopositive nuclei (arrows) were found in neurons of the VMN **(A)** and DMN **(C)** of the hypothalamus. Insets: Higher magnification views of the outlined areas showing immunopositive nuclei (arrow) in VMN **(B)** and DMN **(D)**. III, third ventricle. Bar = 160 μm (A,C); 20 μm (B,D).

times in gestation *(24)*. As serum T concentrations decrease during gestation in the male, cytosolic ARs increase in the hypothalamus and preoptic area *(25)*.

Nuclear ARs have been measured in block-dissected brain areas by receptor binding assays *(26)*. In the gonadally intact fetus on d 125 of gestation, AR seems to be maximally translocated by endogenous androgen. At this gestational age, nuclear AR can be found in the hypothalamus, septum, and preoptic area, although we did not find AR immunostaining in the preoptic area at any gestational age. The differences in these results are likely owing to the level of sensitivity of the binding assays compared to localization of AR by ICC techniques. In particular, binding assays measure AR in brain regions or nuclei, whereas ICC methods localize receptor to individual neurons. Regardless, it is clear that androgens exert important actions in the developing monkey for the organization of sex-specific behaviors in the infant and the adult *(1,2)* and the development of feedback mechanisms for regulating luteotropic hormone (LH) secretion in the fetus *(27)*.

The first indication that LH secretion in the fetal monkey is regulated by androgen was the observation that serum LH concentrations were higher in female compared to male fetuses *(28)*. Later it was demonstrated that gonadectomy of males *in utero* resulted in elevated serum LH concentrations, which could be reduced to intact amounts by treatment with exogenous DHT *(31)*. Because DHT cannot be aromatized, it seems likely that these effects are mediated by an AR mechanism. The approximate time in gestation when androgens begin to exert feedback regulation on gonadotrophin secretion is not known for rhesus monkeys. We do know, however, that sex differences in serum LH concentrations in the fetal monkey have been observed on d 80 of gestation *(28)*. If we assume that the mechanisms for the control of gonadotrophins are similar in the fetus and the adult monkey, then the next logical step might be to consider the role of the ARC (the presumed pulse generator) in feedback regulation of LH in the monkey fetus. Data in this article demonstrate AR immunoreactivity in the ARC on d 124 of gestation and its absence on d 61. These data seem to indicate that ARC lacks AR on d 61 of gestation and that the feedback relationship between androgen and gonadotropic hormone-releasing hormone- (GnRH) producing neurons develop sometime between d 61 and 80 of gestation in this species.

In the adult rhesus monkey, the ARC appears to be responsible for GnRH secretion, because lesions of this brain region inhibit LH release *(29)*. T appears to regulate LH secretion through a hypothalamic mechanism in the monkey in as much as endogenous T cannot control LH secretion in the ARC-ablated animal following GnRH administration *(29)*.

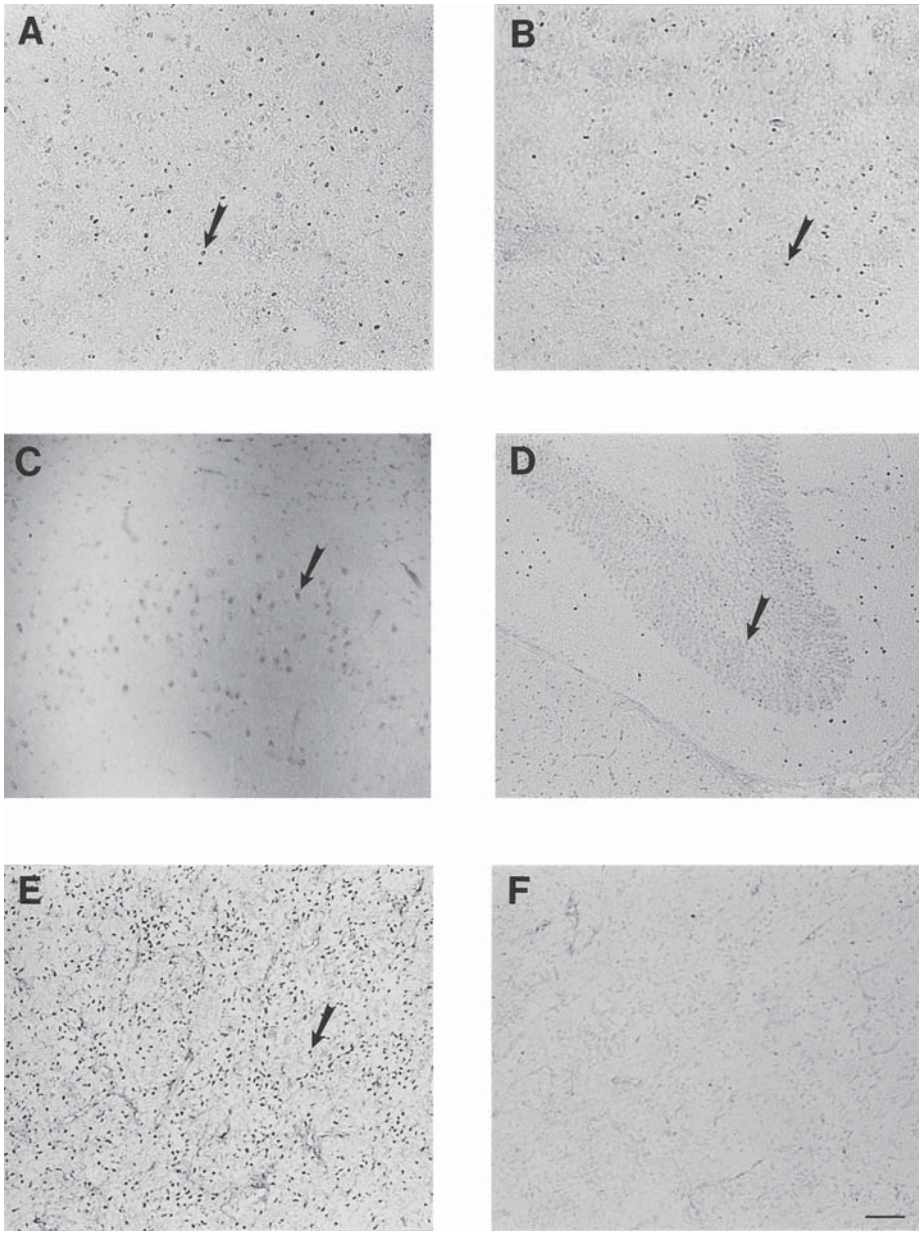

Fig. 4. AR-immunoreactive nuclei (arrows) were found in other brain areas from the adult male, including the lateral hypothalamic area **(A)**, the ARC **(B)** and the CA1-CA3 regions **(C)**, and dentate gyrus of the hippocampus **(D)**. In sections of the anterior pituitary gland, anti-AR antibody stained nuclei of pituicytes (arrow, **E**), but not adjacent sections incubated with preimmune serum **(F)**. Bar = 80 µm.

T secretion by the fetal and adult testes via its receptor may also influence estrogen-dependent developmental events. In adult rats and guinea pigs, androgens regulate brain aromatase activity *(30,31)*. Castration decreases this activity, whereas DHT treatment of castrated animals elevates it to intact levels. This action of DHT occurs through a receptor mechanism *(31)*. In the guinea pig, inhibition of aromatase activity *in utero* affects the development of negative feedback mechanisms, which control LH secretion and gender-specific reproductive behaviors in adulthood *(32)*. In the adult rat, androgens regulate $P450_{AROM}$ mRNA and aromatase activity in many parts of the brain *(33)*. Likewise, aromatase activity in the VMN and the ARC of the adult male cynomolgus monkey is androgen-dependent *(23)*, and there is evidence for selective translocation of AR by androgens in these hypothalamic nuclei *(23)*. In fetal monkeys, however, aromatase activity does not seem to be regulated by androgen *(34)*.

In the adult monkey brain, our data indicate that immunoreactive neurons are localized in the VMN, DMN, lateral hypthalamus, and ARC. These data for the VMH and ARC are similar to those reported previously using ICC *(17)* or androgen autoradiography *(35)*. The data for the DMH and lateral hypothalamus provide new knowledge regarding the distribution of AR in the monkey brain.

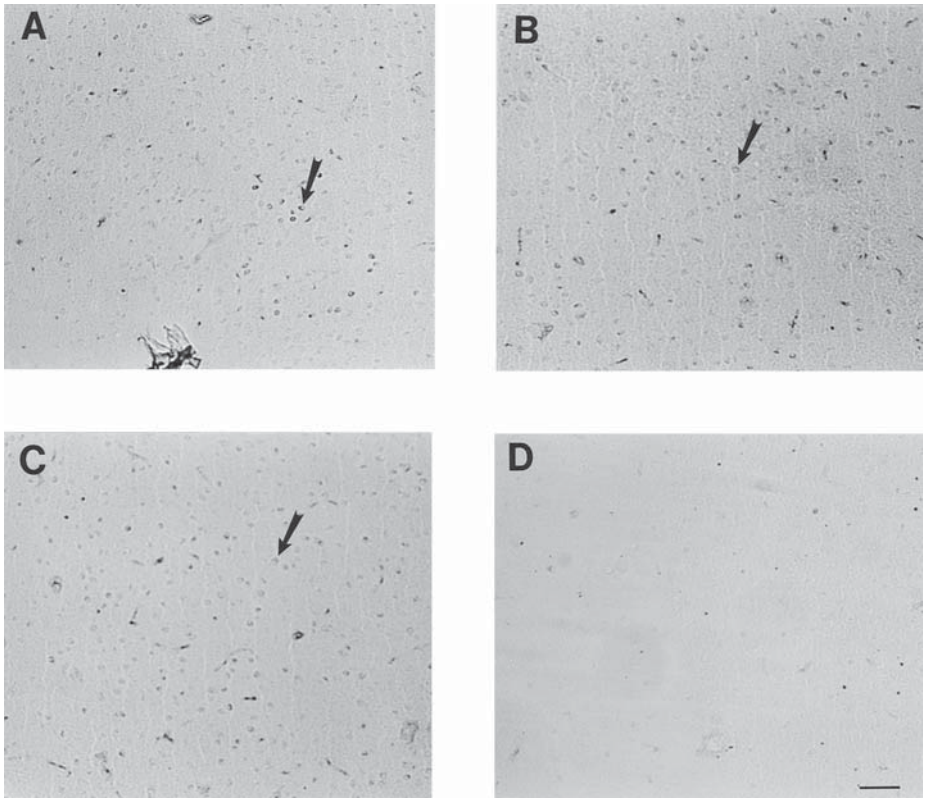

Fig. 5. Temporal lobe sections from the adult male monkey brain stained with anti-AR antibody contained immunopositive nuclei (arrows) in neurons of the medial **(A)**, basal **(B)**, and cortical **(C)** amygdaloid nuclei. An adjacent section stained with antibody preadsorbed with antigen peptide did not show immunostaining **(D)**. Bar = 80 μm.

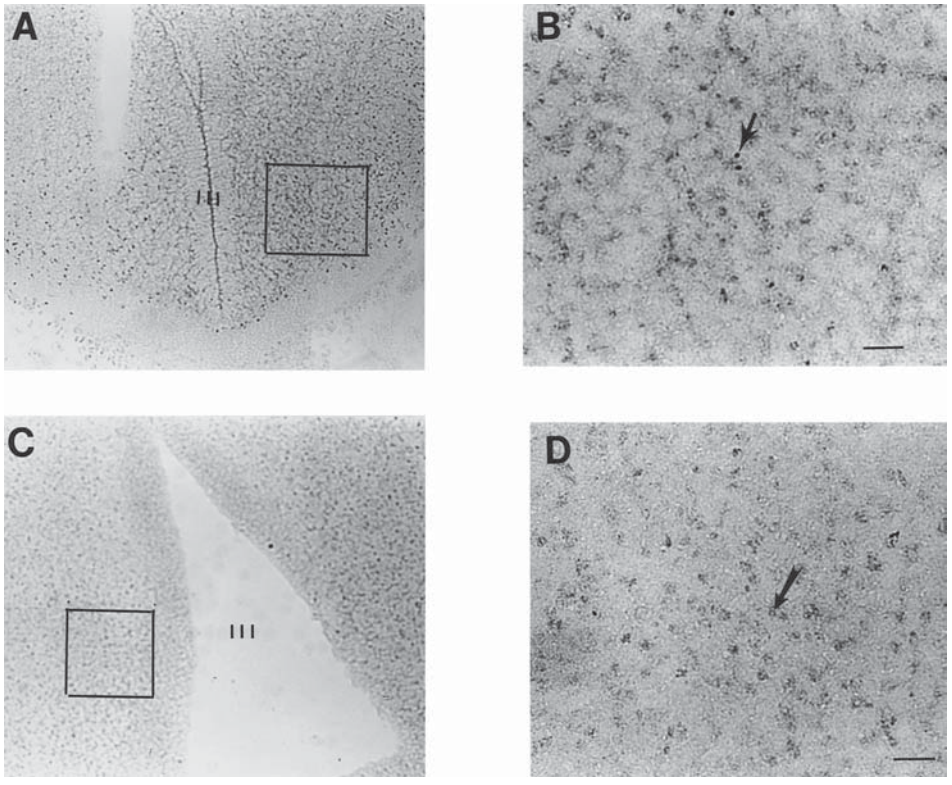

Fig. 6. Sections from the diencephalon of a 47-d fetal male rhesus monkey were stained with anti-AR antibody. Immunopositive nuclei (arrows) were found in neurons of the medial hypothalamic area, excluding the ARC **(A,B)** and the VMN **(C,D)**. III, third ventricle. Bar = 80 μm (A,C) and 20 μm (B, D).

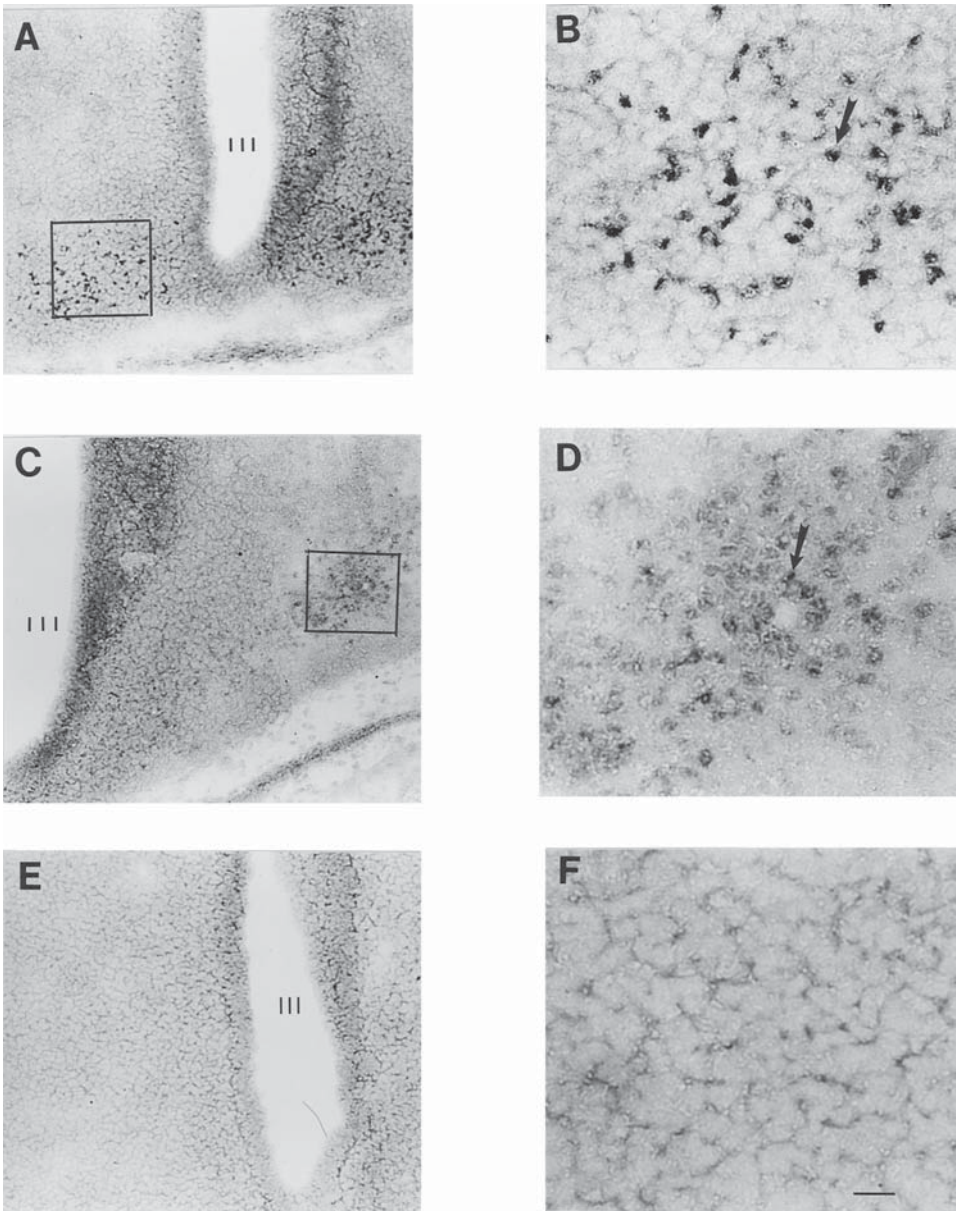

Fig. 7. Sections from the diencephalon of a 61-d fetal male rhesus monkey were stained with anti-AR antibody. Immunopositive nuclei (arrows) were found in neurons in the medial hypothalamic area, excluding the ARC **(A,B)** and the VMN **(C,D)**, but not in the preoptic area **(E,F)**. III, third ventricle. Bar = 80 μm (A,C,E), 20 μm (B,D,F).

Of the brain areas studied, the ARC was the lowest in staining density and the number of AR-positive neurons. Release of LH from the pituitary is subject to feedback regulation by androgens on the medial preoptic area and the ARC. GnRH-containing neurons in the ARC of the rat do not appear to contain estrogen *(36)* receptors or AR *(37)*, however. Likewise, in the ram, AR and GnRH immunoreactivity do not colocalize in hypothalamic neurons *(38)*. These data suggest that steroid effects on GnRH neurons are indirect.

In summary, immunoreactive AR show a distinct distribution in the diencephalon of the fetal male rhesus monkey that is age-dependent. The distribution of AR in the VMN, DMN and ARC by this technique on day 124 of gestation in the male fetus is similar to the distribution of AR in the adult male. Thus, by 124 d of gestation, the cytoarchitecture of AR distribution is already in place. The presence of AR in the cells of the diencephalon probably indicates that androgens act on this region to organize brain areas that will later control reproductive and aggressive behaviors, negative feedback control of gonadotrophins, and, perhaps, other unknown actions.

Materials and Methods

Animals

Animal care throughout these studies was provided by the veterinary staff of the Oregon Regional Primate

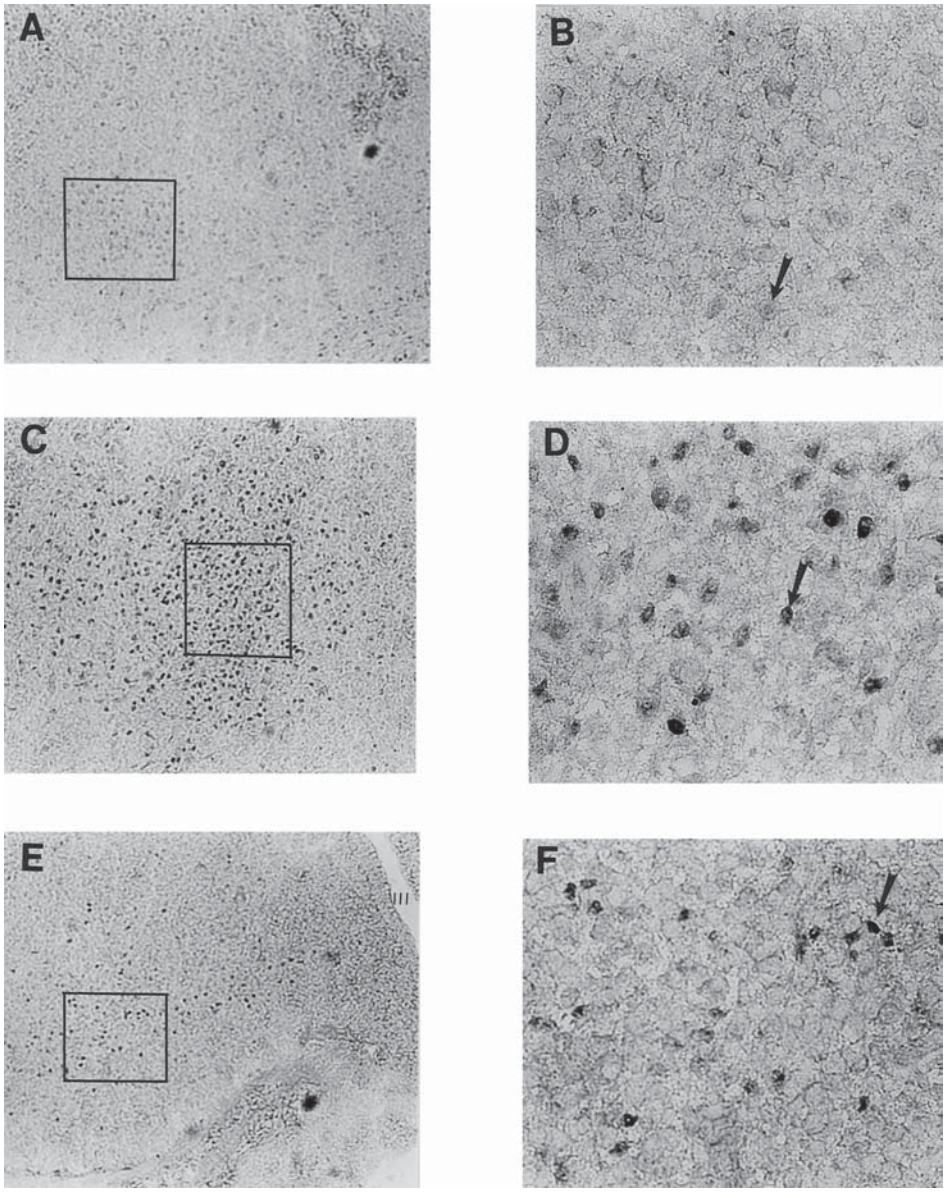

Fig. 8. Sections from the diencephalon of a 124-d fetal male rhesus monkey were stained with anti-AR antibody. Immunopositive nuclei (arrows) were found in neurons in the medial hypothalamic area, excluding the ARC **(A,B)** and the VMN **(C,D)**, and the ARC **(E,F)**. III, third ventricle. Bar = 80 μm (A,C,E), 20 μm (B,D,F).

Research Center (ORPRC) in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Tissues from sexually mature male rhesus monkeys (n = 3) were obtained from the ORPRC Tissue Distribution Program. The monkeys were deeply anesthesized with sodium pentobarbitol (100 mg/kg), exsanguinated, and the brain, prostate, and seminal vesicles of each animal, and the anterior pituitary from one animal were quickly removed and placed on ice. Uterine myometrium from adult female rhesus monkeys was also obtained from the ORPRC Tissue Distribution Program as describe above. The pituitary, the peripheral tissues, and tissue blocks from the diencephalon and the temporal lobes were dissected, then rapidly frozen in a dry ice-chilled isopentane bath, and stored at –80°C.

Male monkey fetuses on d 47, 61, and 124 d of gestation (term, 167 d) were obtained from pregnant female monkeys in the ORPRC Timed Breeding Program. In each case, the monkeys were sedated with 5 mg/kg ketamine and 0.2 mg/kg atropine, and then cesarean delivery was performed under Halothane anesthesia. The deeply anesthesized fetuses were killed by decapitation, and the whole heads of the 47- and 61-d animals were rapidly frozen in a dry ice-chilled isopentane bath and stored at –80°. The diencephalon from the 124-d fetus was obtained by block dissection and rapidly frozen as described above.

AR Antibody

A synthetic peptide corresponding to the first 21 aa of the human androgen receptor was synthesized by the

Molecular Biology Core at the US Veterans Hospital in Portland, OR and had the following sequence: NH_2-MEVQLGLGRVYPRPPSKTYRG-COOH. The peptide was conjugated to bovine thyroglobulin using gluteraldehyde as the coupling agent *(39)* and injected subcutaneously into three New Zealand white rabbits according to the following schedule. On the day of initial immunization, blood (10 mL) was collected for preimmune serum, and 220 nmol of antigen in 1 mL Freund's Complete Adjuvant (Sigma, St. Louis, MO) were injected subcutaneously between the scapulae. Three weeks later, the rabbits received a booster injection containing 220 nmol antigen in 1 mL Freund's Incomplete Adjuvant (Sigma), and the first immune serum (10 mL) was collected 2 wk after this injection and checked for the presence of specific antibodies using a solid-phase dot-blot assay. All subsequent booster injections consisted of 55 nmol antigen in 1 mL Freund's Incomplete Adjuvant administered at 3-wk intervals. Blood (10 mL) was collected 10 d after each treatment. The rabbits were exsanguinated under Nembutal anesthesia 17 wk after the initial immunization. Serum antibodies were affinity-purified as follows: serum (10 mL) was incubated with peptide-conjugated Sepharose 4B beads overnight. The beads were poured into a Bio-Rad Econo-Column (Bio-Rad Laboratories, Richmond, CA), and the column was extensively washed first with 100 m*M* sodium phosphate-buffered saline (PBS) containing 500 m*M* NaCl, then with PBS containing 154 m*M* NaCl. The specifically bound antibody was eluted with 20–25 mL of 100 m*M* glycine, pH 2.1. The antibody solution was dialyzed against 4000 mL PBS for 2 d, concentrated against polyethelene glycol, and used in the immunologic procedures described below.

Sucrose Gradient Shift Assay

Myometrium from a female rhesus macaque was used as a source of primate androgen receptor. Uterine myometrium was separated from endometrium by dissection and homogenized 1:10 (w:v) in TEGMD buffer (10 m*M* Tris, 1.5 mM EDTA, 25 m*M* sodium molybdate, 10% glycerol [v:v], and 1 m*M* dithiothreitol, pH 7.4) using a Brinkman polytron homogenizer (Brinkman Instruments, Westbury, CT). The homogenate was centrifuged for 10 min at 1000*g* at 4°C, and the resulting supernatant was centrifuged for 10 min at 106,000*g* at 4°C. The final supernatant (cytosol) contained unbound AR. Cytosol was incubated for 20 h at 4°C with 3 n*M* [1,2,4,5,6,7-^{3}H]-dihydrotestosterone (^{3}H-DHT, 123 Ci/mmol; NEN Dupont, Wilmington, DE). Affinity-purified polyclonal AR antibody was added to the cytosol incubation 1 h before the cytosol was layered on top of a sucrose gradient. Linear 5–20% sucrose gradients were formed using a Gradient Master™ Model 106 gradient maker (BioComp Instruments Inc., Fredrickton, New Brunswick, Canada). Cytosol was carefully layered on top of the gradient, and the gradient was centrifuged at 205,600*g* overnight at 4°C using a Beckman SW 41 rotor (Beckman Instruments, Palo Alto, CA). The bottom of each ultraclear centrifuge tube (Beckman Instruments) was punctured, and 25-drop fractions collected. The fractions were counted by liquid scintillation spectroscopy, and the amount of ^{3}H-DHT ascertained. Cytosol incubations in which either antiaromatase affinity-purified polyclonal antibody or no antibody was added were used as controls to determine the migration of ^{3}H-DHT-bound AR. We also performed receptor binding assays on aliquots of the myometrial cytosol according to previously published protocols *(25)* using ^{3}H-DHT as the radiolabeled ligand and a 200-fold excess of cold methyltrieneolone (R1881) to determine nonspecific binding.

Western Blot Analysis

Aliquots of rhesus monkey myometrium cytosol (1 mg soluble protein) were loaded onto a discontinuous 10% polyacrylamide gel *(40)*. After separation on the gel, proteins were transferred to a nitrocellulose membrane. Following the transfer, the membrane was incubated in blocking buffer (1.0% nonfat dry milk in 100 m*M* PBS) for 1 h. It was then incubated with affinity-purified polyclonal anti-AR antibody in blocking buffer (10 µg protein/mL diluted 1:200) overnight, followed by peroxidase-conjugated goat antirabbit IgG, according to Vector Kit instructions (Vector Laboratories, Burlingame, CA). The immunoreactive bands were visualized using 0.05% diaminobenzidine intensified with 0.02% cobalt chloride, containing 0.1% hydrogen peroxide. Prestained mol-wt standards (Kaleidescope standards; Bio-Rad) were used to determine the approximate mol wt of the stained bands.

Immunocytochemistry (ICC)

To obtain sections for immunostaining, tissue was mounted on a frozen cryostat chuck with dH_2O. Tissues were equilibrated to –15°C, sectioned at 10 µm on a Leitz 1710 cryostat (Leica Inc., Germany) and thaw-mounted onto chrome alum-gelatin coated microscope slides. Sections, air-dried for 4–5 min, were still moist when they were fixed in 4.0% paraformaldehyde in 100 m*M* phosphate buffer for 5 min. The tissue was washed twice in 100 m*M* PBS and incubated in 1.0% H_2O_2 in PBS for 10 min, followed by a single 5-min PBS wash. The tissue was blocked with normal goat serum diluted 1/100 in PBS. Primary antibody was diluted to 7 µg protein/mL in PBS containing 1.0% Triton X-100, 1.0% normal rabbit serum, and 0.1% gelatin. The tissue sections were incubated for 40 h with the primary antibody at 4°C in a humidified chamber. The tissue was washed twice with PBS and incubated with biotin-conjugated goat antirabbit secondary antibody according to the directions provided with the Vector Elite ABC Kit (Vector Laboratories, Inc.) for 1 h, washed twice with PBS, and then incubated with Vector peroxidase-conjugated avidin–biotin complex for 1 h following kit instructions. The sections were washed with PBS for 5 min followed by a 5-

min wash in 50 m*M* Tris-HCl buffer, pH 7.6. AR specific cells were visualized using 0.025% diaminobenzidine dissolved in 50 m*M* Tris-HCl buffer containing 0.01% H_2O_2. All steps subsequent to the primary antibody incubation were performed at 20°C. Adjacent sections were counterstained with luxol fast blue and neutral red to facilitate anatomic localization of AR-specific cells using published primate brain atlases *(41,42)*.

Preimmune and antiaromatase sera were, also, purified by affinity chromatography. These preparations were used as controls for ICC and the sucrose gradient shift assay, respectively, in the same concentrations as affinity-purified AR antibody.

Acknowledgments

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