ANDROGEN RECEPTOR-LIKE IMMUNOREACTIVITY IN THE DEVELOPING BRAZILIAN OPOSSUM BRAIN AND PITUITARY: ONTOGENY AND EFFECTS OF EXOGENOUS TESTOSTERONE ADMINISTRATION

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ABSTRACT

Indirect immunohistochemistry and an androgen receptor antibody, PG-21, was used to characterize the ontogeny of cells containing androgen receptor-like immunoreactivity (AR-ir) and the effects of exogenous testosterone administration on AR-ir in the developing Brazilian opossum brain. Cells containing AR-ir were first seen in the anterior pituitary at day 5 of postnatal life (PN). Between 10 and 15 PN, high numbers of immunoreactive cells were detected in the dorsomedial hypothalamic and ventral premammillary nuclei, and a few cells were seen in the arcuate nucleus of the male brain. The female brain of the same age had a low number of moderately immunostained cells in the dorsomedial hypothalamic and ventral premammillary nuclei. No AR-ir was observed in other areas of the brain until 45 PN, when a low number of immunoreactive cells were seen in the ventral nucleus of the lateral septum of the male. In males, between 60 and 80 PN, cells containing AR-ir were present in additional areas of the forebrain and resembled that of the adult. In the midbrain and brainstem, AR-ir was first seen at 80 PN in the male in the central gray, mesencephalic trigeminal nucleus, medullary reticular formation, and the nucleus of the solitary tract. In the female, no AR-ir was seen in any of these areas at 60 and 80 PN. Exogenous testosterone injection 2 hours prior to tissue collection did not result in any change in AR-ir in animals at 1, 3, and 5 PN. However, between 10 and 15 PN, testosterone exposure resulted in an increase in the number and intensity of AR-ir in cells in the dorsomedial hypothalamic nucleus, arcuate nucleus, arcuate-median eminence region, ventral premammillary nucleus, and anterior pituitary gland. Testosterone treatments also resulted in the expression of AR-ir in cells in several of the forebrain and midbrain areas in both sexes. Testosterone injection at 25 PN resulted in expression of AR-ir in cells in similar brain areas as was observed at 15 PN in both the male and female. These findings suggest that the sex differences in AR-ir observed under physiological conditions in the opossum brain are possibly due to differences in plasma androgen levels in the male and female during postnatal development. Further, androgen receptors are present in several brain areas of the opossum during early postnatal development, which can be detected immunohistochemically using PG-21 after exogenous testosterone administration. The early presence of androgen receptors indicate that androgens may be involved in sexual differentiation and neuroendocrine regulation in the opossum brain. Further work will define the significance of these results.

INTRODUCTION

Androgens play an important role in the development and sexual differentiation of the mammalian brain (Gorski and Jacobson, 1982). For example, development of the sexually dimorphic nucleus of the preoptic area (SDN-POA) is under the influence of androgens during the critical period of brain development in the rat (Jacobson *et al.*, 1981; Dohler *et al.*, 1984). Presently, data indicate that there are species differences in regard to whether estrogens formed after aromatization or the androgens themselves are directly involved in the process of masculinization and/or defeminization of the brain. In rodents sexual differentiation of the brain and regulation of adult sexual behavior is dependent upon aromatization of androgens to estradiol (Gorski and Jacobson, 1982; Arnold and Gorski, 1984; Shinoda *et al.*, 1994), whereas in non-human primates and guinea pig, androgens act directly to masculinize and defeminize

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the brain and affect sexual and reproductive behaviors (Connolly and Resko, 1994; Connolly *et al.*, 1994).

During development and in adulthood a variety of events including neurite growth, synaptogenesis, growth and development of motoneurons, synaptic morphology and spine density, and processing of somatosensory and chemosensory information all require gonadal steroids for their regulation (Shughrue and Dorsa, 1993; 1994). Gonadal steroids have also been shown to modulate and influence the expression of various neuropeptides in the brain, and subsequently regulate centrally mediated functions. For instance, immunohistochemical studies have demonstrated that cholecystokinin (Simerly and Swanson, 1987; Micevych *et al.*, 1988; Fox *et al.*, 1991c), arginine vasopressin (De Vries *et al.*, 1983; 1985), substance-P (Malsbury and McKay, 1987; 1989), and monoamine and catecholamine systems (Stewart *et al.*, 1991; Sterwart and Rajbi, 1994) each are sexually dimorphic, and their expression is influenced by the levels of circulating gonadal hormones during development.

The exact mechanism through which gonadal steroids and specifically androgens are involved in sexual differentiation of the brain is not completely understood. It is generally believed that gonadal steroids act as signal transduction molecules, neurotrophic factors, and/or neuromodulators through their specific intracellular receptors (Miesfeld, 1989; Walters, 1985). After binding to their receptor, androgens alter gene expression and/or transcriptional factors in the cell (Yamamoto, 1985).

The neuroanatomical distribution of androgen receptors has been reported in the adult brain of a number of species (Choate and Resko, 1992; Clancy *et al.*, 1992; 1994; Simerly *et al.*, 1990). In addition, studies using in *vitro* binding assays have demonstrated the existence of cytosolic and nuclear androgen receptors in the hypothalamic preoptic area during the critical period of brain development in non-human primates, guinea pigs, and rodents (Attardi and Ohno, 1976; Fox *et al.*, 1978; Handa *et al.*, 1988; Toyoka *et al.*, 1989; Vito and Fox, 1982). However, the development of androgen receptors has not been immunohistochemically characterized in any mammalian brain.

To this end, we have utilized the Brazilian gray short-tailed opossum, *Monodelphis domestica*. *Monodelphis* is a small pouchless marsupial and its young are born after 14 days of gestation in an extremely immature and sexually undifferentiated state before neurogenesis and organogenesis are completed (Iqbal et al., 1995a; Kuehl-Kovarik et al., 1995). Previously, we have reported the ontogeny and distribution of cells containing estrogen receptor-like immunoreactivity (Fox *et al.*, 1991b;c). We have also examined the distribution of androgen receptor-like immunoreactivity and the effects of castration and testosterone replacement in the adult male Brazilian opossum brain (Iqbal *et al.*, 1995b). In the present study, we have taken advantage of the unique reproductive physiology of Monodelphis to examine the ontogeny of cells containing androgen receptor-like immunoreactivity (AR-ir) in the brain of developing male and female Brazilian gray short-tailed opossums. Since the antibody we have chosen to use preferentially recognizes bound (nuclear) androgen receptors, we also examined the effects of exogenous testosterone administration on androgen receptor expression in the developing opossum brain.

MATERIALS AND METHODS

Study 1

To characterize the ontogeny of cells containing androgen receptor-like immunoreactivity, developing male and female Brazilian gray short-tailed opossums of various ages from postnatal day 1 through 80 were utilized. All animals were obtained from a colony being maintained at Iowa State University. The adult animals were housed individually in plastic cages and maintained on a 14:10 hours light dark cycle with a constant temperature (26°C). Food and water were freely available to the animals (Reproduction Fox Chow; Milk Specialties Products, Madison, WI). All procedures and protocols used in the study have been approved by the Iowa State University Committee for Animal Care.

To obtain postnatal animals, pairs of adult female and male animals were housed together for two weeks. The animals were then separated, and the females checked daily for the birth of pups. The gestation period of the Brazilian opossum is between 14 and 15 days postfertilization. The day of birth of the pups was designated as day 1 of postnatal life (PN). The gender of the pups cannot be identified grossly before the age of 5 PN, thus pups prior to 5 PN were considered sexually undifferentiated. Four pups were investigated at each of the ages of 1, 3, and 5 PN, and at later ages, six animals (3 male and 3 female) from at least three different litters were used at each 10, 15, 25, 35, 45, 60, and 80 PN.

Study 2

To examine the effects of exogenous testosterone administration on AR-ir in the developing opossum brain at 1, 3, 5, 10, 15, and 25 PN animals were injected subcutaneously with testosterone (Sigma: 1

μg/gm body weight; dissolved in sesame oil) or with sesame oil which served as the control. Four pups were used at each of the ages of 1, 3, and 5 PN. Three pups were injected with testosterone and one pup received an injection of sesame oil of equal volume. At 10, 15, and 25 PN, four pups (2 males and 2 females) received a testosterone injection and four pups (2 males and 2 females) were exposed to oil. Animals were killed 2 hours after the injection and processed as described below. At 15 PN, four additional pups (2 males and 2 females) were killed 15 minutes after the testosterone injection.

Tissue collection

The brains were collected as has been described previously (Fox *et al.*, 1991 a;b). Briefly, the brains from developing opossum pups (1, 3, 5, 10, 15, 25 PN) were collected by cooling animals at -15°C until anesthetized. The heads were removed by decapitation and immersed in Zamboni's fixative solution for 48 hours at room temperature. After fixation, the heads were transferred into 30% buffered-sucrose solution overnight, and then cut into 20 μm thick coronal sections on a cryostat (Reichert), thaw mounted onto poly-L-lysine coated slides (Sigma), air dried overnight, and stored at 4°C until immunohistochemistry was conducted.

Animals of 35 and 45 days of postnatal age were anesthetized by cooling and were perfused transcardially with 15 ml of Zamboni's fixative solution. The brains were removed and postfixed in the same manner for 48 hours at room temperature. After postfixation, the brains were processed as described for the younger animals. The brains from 60 and 80 PN animals were collected as has been described previously (Fox *et al.*, 1991b). The animals were anesthetized with ether and perfused transcardially through the left ventricle with Zamboni's fixative solution for 15 minutes. The brains were removed and postfixed for 48 hours and sunk in 30% sucrose solution. The brains from 60 PN animals were processed as described above. The brains of 80 PN animals were cut into 40 μm thick coronal sections and collected into cryoprotectant solution and stored at -15°C for immunohistochemistry.

Immunohistochemical procedures

Immunohistochemistry

To identify cells expressing AR-ir in the developing opossum brain, we have used the androgen receptor antibody, PG-21. Its detailed isolation, characterization (Prins *et al.*, 1991) and use as an immunological probe for androgen receptors in the opossum brain has been reported previously (Iqbal *et al.*, 1995).

The protocol utilized for immunohistochemistry was a modification of that which has been reported previously (Fox etal., 1991b;c). The slide mounted sections were washed in potassium phosphate-buffered saline solution followed by a 30 minute incubation in 0.3% hydrogen peroxide solution. After blocking in normal goat serum (Vector; 1:67), the tissue sections were incubated in PG-21 (3 μg/ml) in a humidified chamber for 48 hours at room temperature. After adequate rinsing with buffer, the tissue sections were incubated in goat anti-rabbit IgG (Vector; 1:200) for 2 hours, rinsed, and exposed to avidin-biotin complex (Vector Elite Kit; 1:50) for one hour in a humidified chamber at room temperature. After washing, the tissue sections were stained by reacting with a substrate composed of 0.04% 3,3' diaminobenzidine tetrahydrochloride (DAB; sigma), 2.5% nickel sulfate (Fisher Scientific), and 0.01% hydrogen peroxide, dissolved in 0.1 M sodium acetate solution. After staining for 8 minutes, the tissue sections were washed in 0.9% saline solution two times to terminate the reaction.

Additional tissue sections from 15 PN male and female animals that were processed for androgen receptor immunohistochemistry were incubated in DAB substrate for 60 minutes to detect cytoplasmic staining for androgen receptors as reported by Wood and Newman (1993). The tissue sections were dehydrated in an ascending series of alcohols, cleared in xylene, and coverslipped with permount mounting media. The tissue sections from 80 PN animals were processed for immunohistochemistry utilizing a free floating tissue technique reported previously (Fox *et al.*, 1991c).

Immunohistochemical control procedures

The details of immunohistochemical control procedures performed to validate the use of PG-21 antibody in the opossum were reported earlier (Iqbal *et al.*, 1995).

Analysis of tissue

Brain sections at 60 μm intervals from areas caudal to the olfactory bulbs to the caudal brainstem from 1 to 60 PN animals and at 160 μm intervals from 80 PN animals were examined with a light microscope. Brain regions containing AR-ir were identified with the aid of maps of coronal sections of the opossum brains published previously (Fox *et al.*, 1991a;b) and an atlas of the developing rat nervous system (Paxinos *et al.*, 1994). For the purpose of discussion, the number of cells with AR-ir in a region were arbitrarily classified

as none: few, if there were single scattered labelled cells; high, if there were >100 labelled cells in the field/ area under view at 40x; and low, if the number of cells with AR-ir was intermediate to the few and high ratings.

RESULTS

The antibody, PG-21 readily recognized cells containing AR-ir in the developing opossum brain and anterior pituitary gland. The androgen receptor immunostaining was limited to the nucleus of the cell. No AR-ir was seen in the tissue sections processed for immunohistochemical control procedures. Thus immunostaining observed in the developing opossum brain and anterior pituitary gland appear to be specific for androgen receptors.

The brain of the neonatal opossum at birth is very immature, and undergoes very rapid developmental and morphological changes during the first two weeks of postnatal life. The specific nuclear groups are easy to recognize using the light microscope by 15 PN. Before 15 PN, the nuclear groups discussed are undergoing developmental changes and are considered presumptive.

AR-ir in the developing opossum brain

Postnatal days 1-5

No AR-ir was seen in the developing opossum brain between 1 and 5 PN. However, low numbers of evenly distributed cells with AR-ir were first seen at 5 PN in the developing anterior pituitary gland.

Postnatal days 10-15

A moderate number of cells containing androgen receptor immunostaining were first detected in the brain of the male animals in the dorsomedial hypothalamic nucleus (Fig. 1 A) and ventral premammillary nucleus at 10 PN. Few cells were seen in the arcuate nucleus (Fig. 1 A) and posterior hypothalamic area. The anterior pituitary gland showed an increase in the number of immunoreactive cells (Fig. 1 C). By 15 PN, a slight increase was observed in the number of cells containing AR-ir in these areas. A few cells were also seen in the arcuate-median eminence region at 15 PN of male animals.

Brains of 10 and 15 PN females had a moderate number of immunostained cells in the dorsomedial hypothalamic nucleus and ventral premammillary nucleus (Fig. 1 B). No AR-ir was seen in cells in the arcuate nucleus and arcuate-median eminence area in the female brain (Fig. 1 D). Androgen receptor-like immunoreactive cells were present in the anterior pituitary gland of the female animals at 10 and 15 PN (Fig.1 D).

Postnatal days 25-45

In the male, the dorsomedial hypothalamic and ventral premammillary nuclei (Fig. 2 A) contained a high number of cells expressing AR-ir and a moderate number of immunoreactive cells were present in the arcuate nucleus and arcuate-median eminence region at this age (Fig. 2 A, Fig. 3 A). Few cells containing AR-ir were also seen in the dorsal division of the premammillary nucleus. The anterior pituitary gland of the male had an increased number of cells expressing AR-ir (Fig. 3 A). There was no apparent change in AR-ir in these regions in the male between 25, 35 and 45 PN. However, very light immunoreactivity was seen for the first time in the ventral nucleus of the lateral septum at 45 PN.

In the female, a slight increase was seen in the number of cells and intensity of immunostaining in the dorsomedial hypothalamic and ventral premammillary nuclei between 25 and 35 PN (Fig. 2 B). The anterior pituitary gland had a slight increase in number of immunopositive cells (Fig. 3 B). No AR-ir was seen in the arcuate nucleus (Fig. 2 B; Fig. 3 B) or in any other areas of the femal brain.

The androgen receptor immunostaining in the male animals was mostly limited to the nucleus of the cell (Fig. 2 A) whereas in the female animals the immunostaining appeared to be both nuclear and cytoplasmic (Fig.2 B).

Postnatal days 60-80

In the male between 60 and 80 PN, AR-ir in cells was seen in several areas of the brain. In the forebrain, a moderate number of cells with AR-ir was present in the ventral nucleus of the lateral septum (Fig. 4 A). The dorsal and intermediate nuclei of the lateral septum each contained a few immunoreactive cells. The medial preoptic area, lateral preoptic area, stria terminalis, and bed nucleus of the stria terminalis each contained a few to a low number of cells expressing AR-ir (Fig. 4 C). The medial and central nuclei of the amygdala and the nucleus of the lateral olfactory tubercle contained a low numbers of cells containing Ar-ir (Fig. 4 E). Few cells were also seen in the posterior hypothalamic area, nucleus of the fields of Forale, and dorsal premammillary nucleus. There was also a slight increase in the number of immunoreactive cells in the ventral premammillary nuclei compared to what was

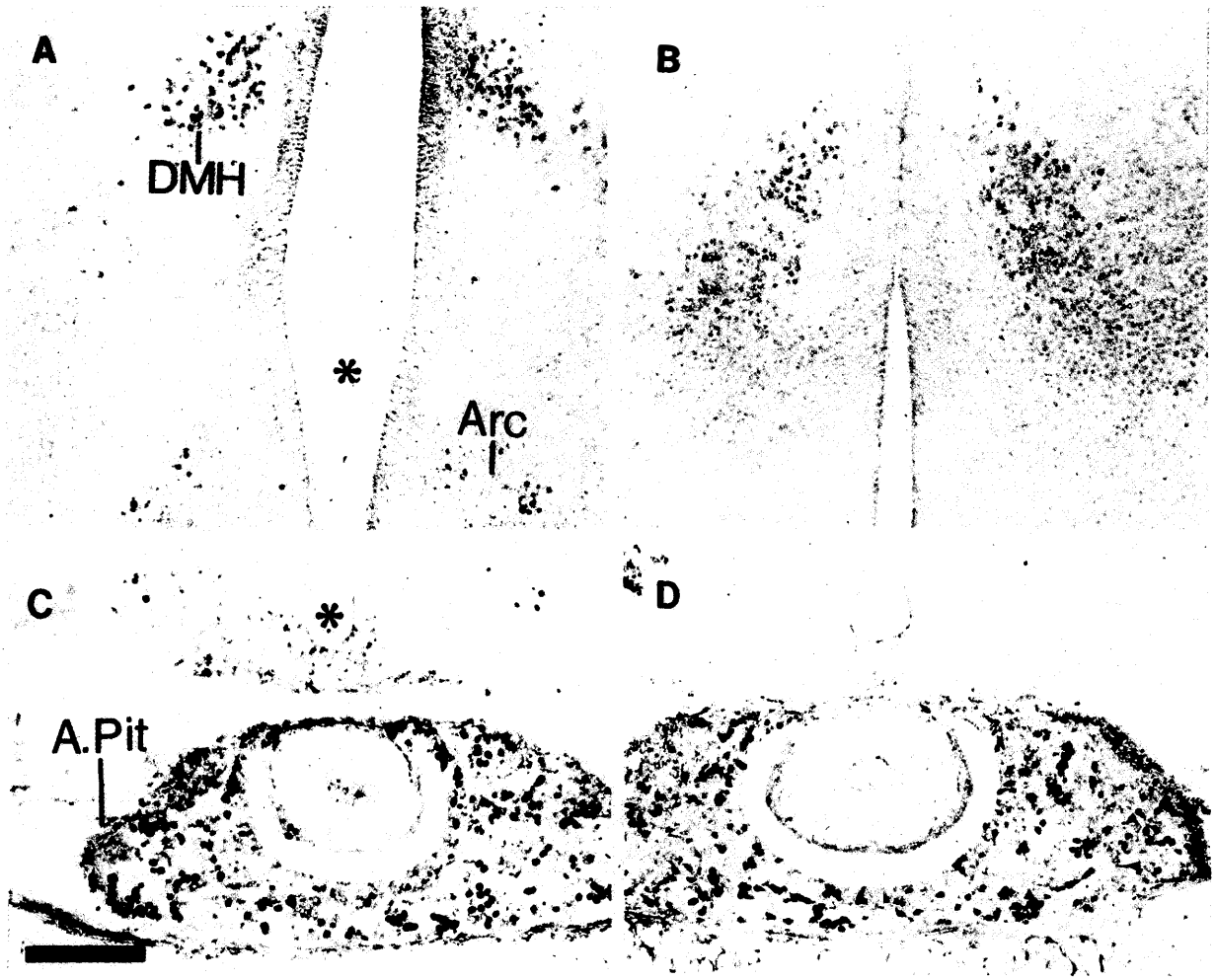

Fig. 1: Photomicrographs of coronal sections from the brain and anterior pituitary gland demonstrating androgen receptor-like immunoreactivity in cells in the dorsomedial hypothalamic nucleus (DMH: A,B) and anterior pituitary gland (A.Pit; C,D) of the 10 PN male (A,C) and female (B,D). Few immunoreactive cells are seen in the arcuate nucleus (Arc) of the male (A) whereas no immunoreactivity is seen in the arcuate nucleus in the female (B). The asterisk indicates the third ventricle. All photomicrographs are at the same magnification. Scale bar= 100 μm

seen at 45 PN. In the midbrain, a few cells containing AR-ir were seen in the central gray and mesencephalic trigeminal nucleus. In the brainstem, a few cells with AR-ir containing cells were seen in the medullary reticular formation and nucleus of the solitary tract.

No cells containing AR-ir were seen in any of the areas of the brain of females (Fig. 4 B,D,F) between the ages of 60 and 80 PN in which AR-ir was observed in the male. Like previous ages, the anterior pituitary gland of the male had higher numbers of immunoreactive cells and a more intense immunoreactivity as compared to the anterior pituitary gland of the female of the same age.

Effects of exogenous testosterone administration

No effects of testosterone injection were observed in the brain or anterior pituitary gland of 1, 3 and 5 PN animals. Testosterone injection at 10 PN, resulted in an increase in the intensity of immunoreactivity and in the number of cells containing AR-ir in the dorsomedial hypothalamic (Fig. 5 A,B) and ventral premammillary nuclei, and anterior pituitary gland (Fig. 5 C,D) in both male and female animals. A slight increase was seen in the arcuate nucleus of the male brain (Fig. 5 A), whereas, a few immunoreactive cells were observed in the arcuate nucleus of the female brain also at this time (Fig. 5 B). Lightly immunostained cells were also seen

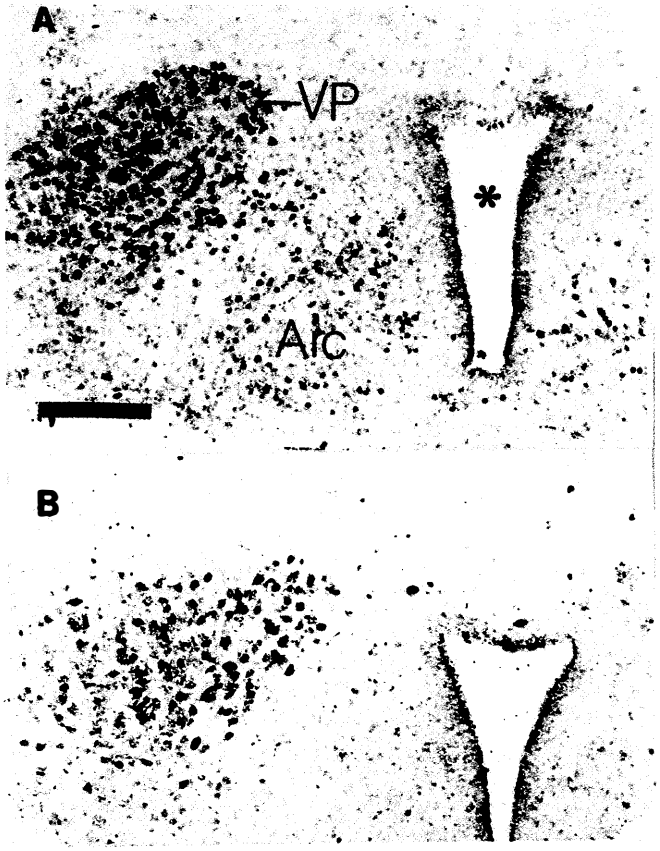

Fig. 2. Photomicrographs of coronal sections through the 25 PN opossum brain demonstrating androgen receptor-like immunoreactivity in the arcuate-median eminence region and ventral premammillary nuclei of the male (A) and in the ventral premammillary nucleus of the female (B). Note the absence of immunoreactivity in the arcuate nucleus of the female (B). Scale bar in A,B = 100 μm.

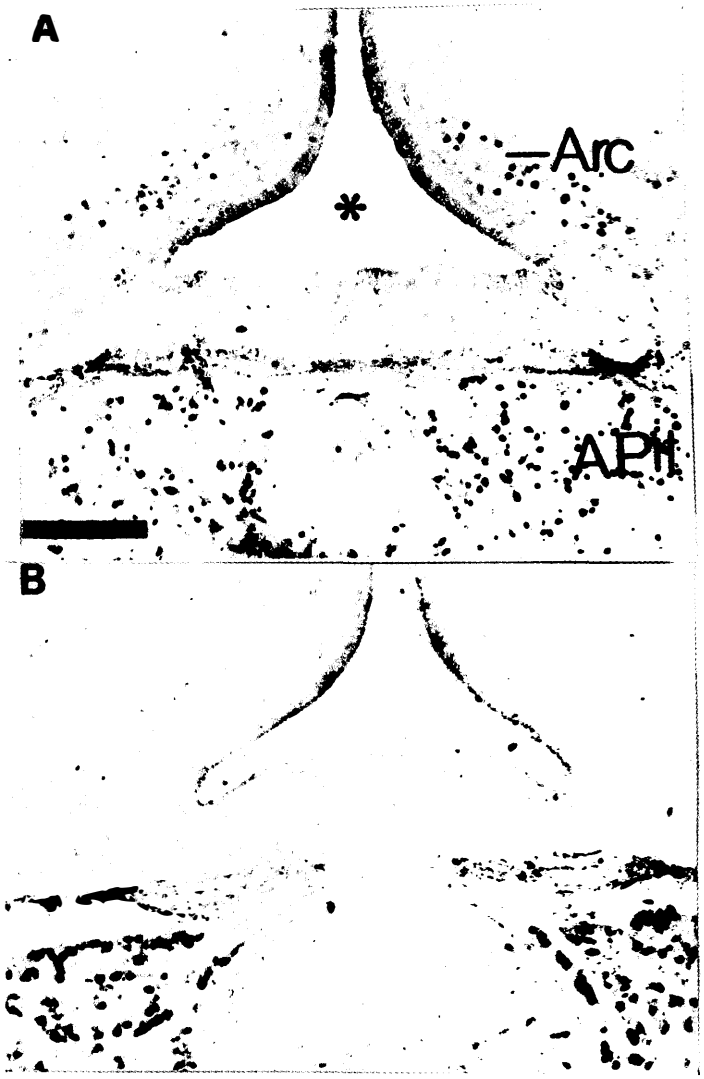

Fig. 3. Immunohistochemical demonstration of androgen receptor-like immunoreactivity in cells in the arcuate nucleus (Arc) and anterior pituitary gland (A.Pit) of the 25 PN male (A). In the 25 PN female, no androgen receptor-like immunoreactivity was seen in the arcuate nucleus (B), whereas immunostaining was present in the anterior pituitary gland. The asterisk indicates the third ventricle. Both photomicrographs are at the same magnification. Scale bar= 100 μm

in the forming lateral preoptic area (Fig. 6 A), medial amygdala (Fig. 6 B), ventromedial hypothalamic nucleus (Fig. 6 C), supramammillary nucleus, ventral tegmental area, and pontine central gray in both sexes.

In 15 PN animals which received testosterone injection 15 minutes before decapitation, there was no change in AR-ir in the male and female brain except for a slight increase in immunostaining in the anterior pituitary gland of both sexes. Testosterone injections in 15 PN males 2 hours before decapitation resulted in an increase as copmared to uninjected or control 15 PN males in the number of immunoreactive cells and intensity of immunostaining in the dorsomedial hypothalamic nucleus, arcuate nucleus (Fig. 7 A), arcuate-median eminence region, ventral premammillary nucleus, and anterior pituitary gland (Fig. 7 C). The expression of AR-ir in these areas in the female brain after testosterone injection resembled that of the male brain (Fig. 7 B,D).

A low to moderate number of androgen receptor immunoreactive cells were observed in several additional brain areas both in the male and female,

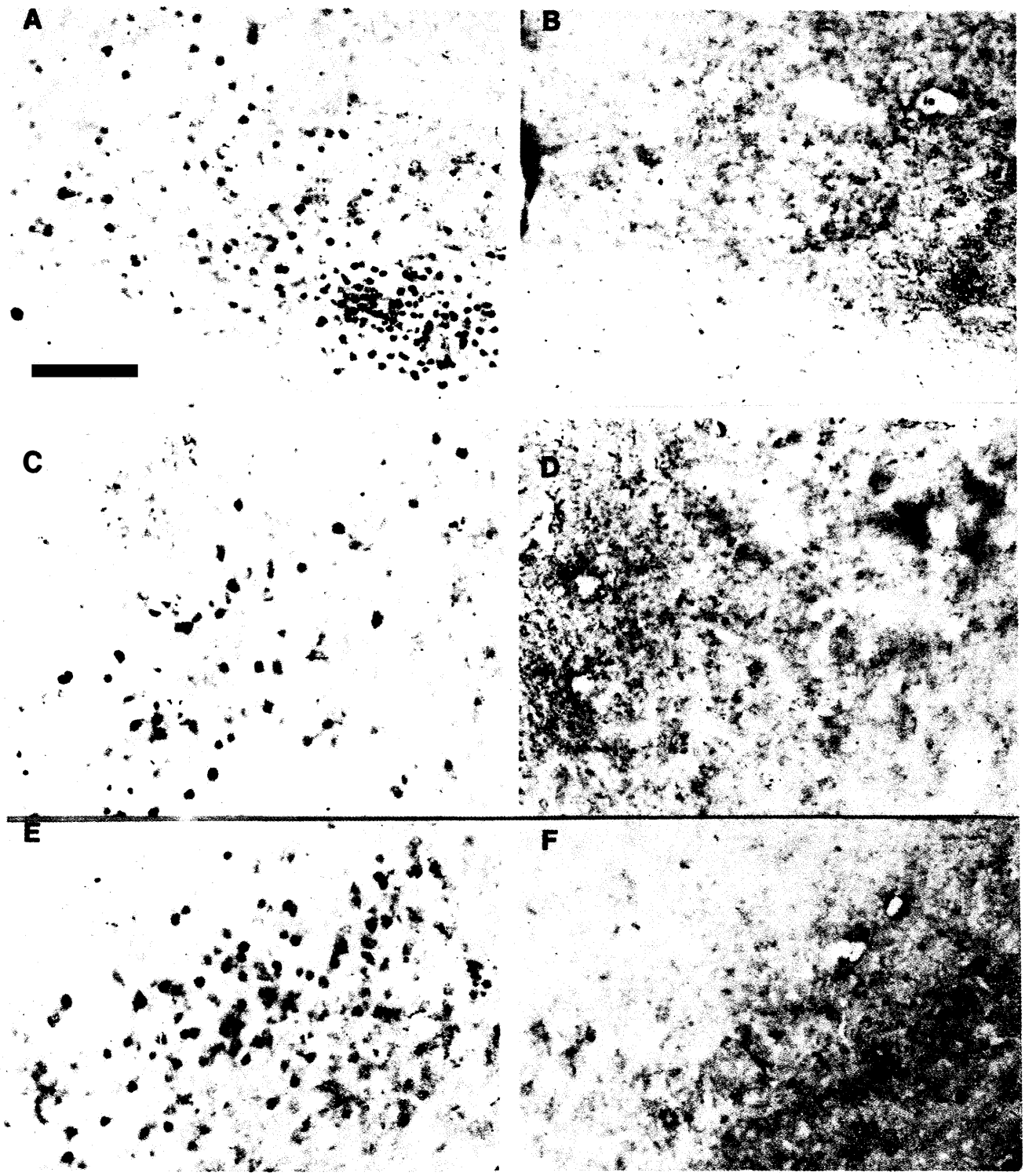

Fig. 4. A series of photomicrographs of coronal sections from the brain of the 60 PN male (A,C,E) and female (B,D,F) opossum demonstrating differences in the expression of androgen receptor-like immunoreactivity in the lateral septum (A and B), lateral preoptic area (C and D), and medial amygdala (E and F). Note the absence of androgen receptor-like immunoreactivity in cells in the female brain. Medial is towards the right in all photomicrographs. All photomicrographs are at the same magnification. Scale bar = 100 μm

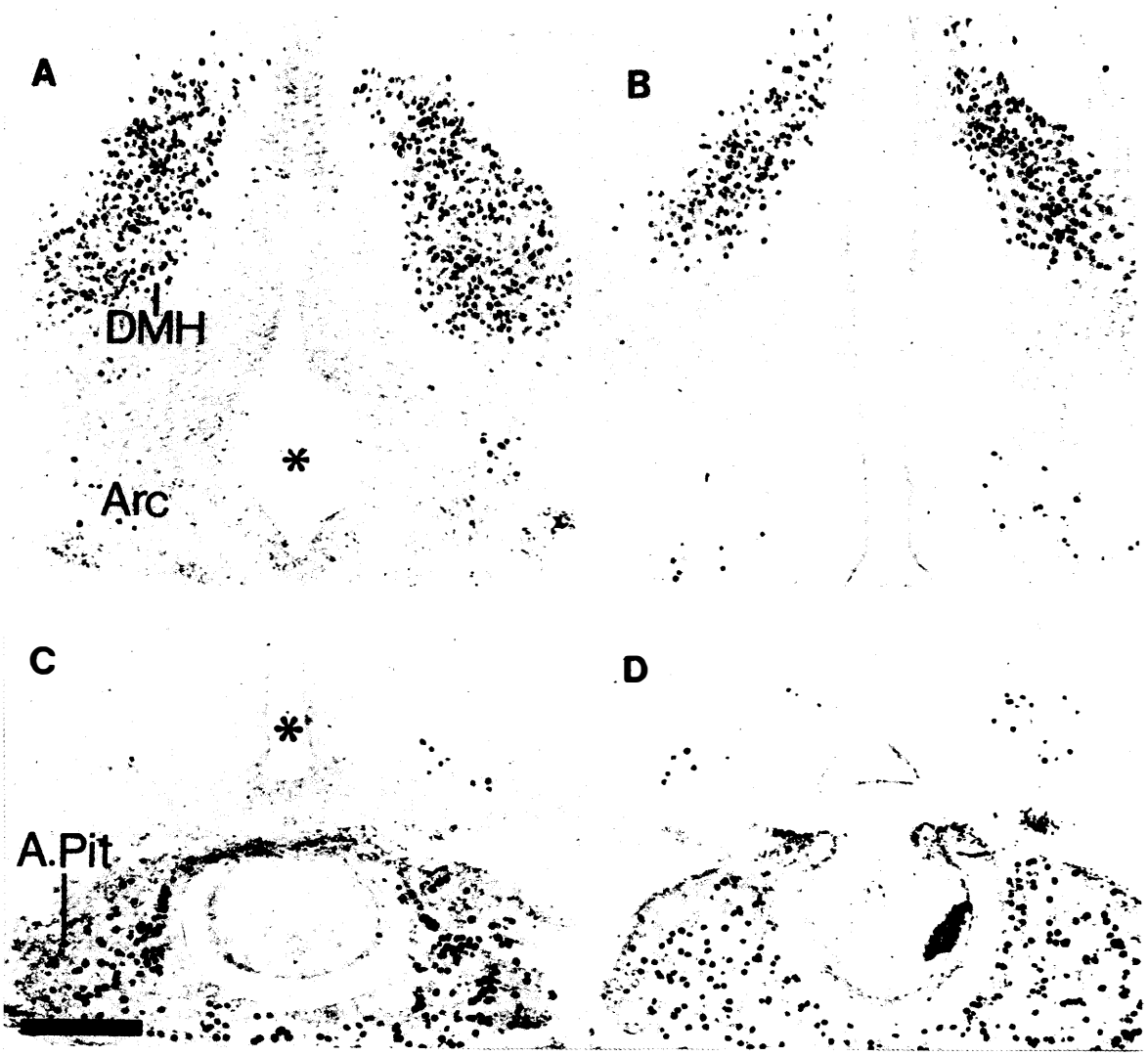

Fig. 5. Photomicrographs demonstrating the effects of testosterone administration in the 10 PN male (A,C) and female (B,D). Testosterone injection resulted in an increase in androgen receptor-like immunoreactive cells in the dorsomedial hypothalamic nucleus (A,B; DMH) and anterior pituitary gland (C,D; A.Pit) both in the male and female. A few immunoreactive cells were also present in the arcuate nucleus (Arc) in the male (A) and female (B). The asterisk indicates the third ventricle. All photomicrographs are at the same magnification. Scale bar= 100 μm

including the lateral septum (Fig. 8 A), stria terminalis, median preoptic nucleus, medial and lateral preoptic areas (Fig. 8 A), bed nucleus of the stria terminalis, subfornical organ, vascular organ of the lamina terminalis, suprachiasmatic nucleus (Fig. 8 B), and ventral tegmental area (Fig. 8 C). A few cells containing AR-ir were also present in the ependyma of the lateral and third ventricles.

There was no change in the expression of AR-ir in the male and female animals which received injection of vehicle. Testosterone administration in 25 PN animals resulted in the detection of cells containing AR-ir in similar brain areas in the male and female as was observed in the 15 PN animals.

DISCUSSION

In the present study, we have used the androgen

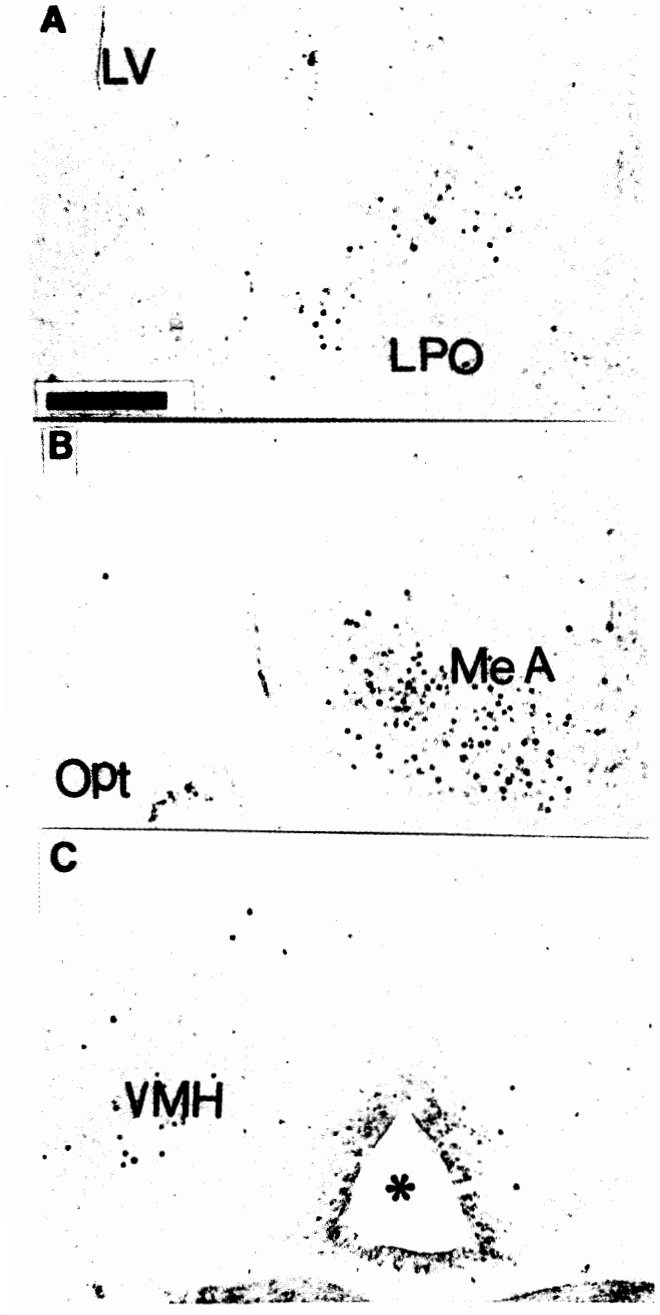

Fig. 6. Photomicrographs of coronal sections through the brain of a 10 PN male demonstrating the expression of androgen receptor-like immunoreactivity in the forming lateral preoptic area (LPO; A), medial amygdala (MeA; B), and ventromedial hypothalamic nucleus (VMH; C) after testosterone injection. The asterisk in C indicates the third ventricle. Abbreviations; LV, lateral ventricle; Opt, optic tract. All photomicrographs are at the same magnification. Scale bar = 100 μm

receptor antibody, PG-21, to examine the developmental changes in AR-ir in the developing opossum brain. This antibody has been extensively characterized and its use as an immunological probe for androgen receptors in brain has been demonstrated (Clancy *et al.*, 1994; Prins *et al.*, 1991; Wood and Newman, 1993; Zhou *et al.*, 1994). The use of the PG-21 antibody in opossum tissue was validated using various control procedures and have been described in detail elswhere (Iqbal *et al.*, 1995). Results of control procedures demonstrated that immunostaining observed in the developing opossum brain and anterior pituitary gland is specific for androgen receptors.

Cells expressed AR-ir as early as 5 PN in the anterior pituitary gland, an age when the gender of the opossum pups can not be recognized grossly. In the male brain, AR-ir was seen in the dorsomedial hypothalamic nucleus, arcuate nucleus, arcuate-median eminence, posterior hypothalamic area, and ventral premammillary nucleus between 10 and 15 PN, whereas the female brain had moderate immunostaining only in the dorsomedial hypothalamic and ventral premammillary nuclei at this age. A low number of moderately stained cells were seen in the ventral nucleus of the lateral septum at 45 PN in the male. Between 60 and 80 PN, AR-ir in the male brain was seen in several additional areas of the forebrain, and at 80 PN, AR-ir in cells was also detected in the midbrain and brainstem. Conversely, no AR-ir was seen in additional areas in the females between the ages of 60 and 80 PN. The distribution of androgen receptors has been reported in similar areas in the brain of the adult male rat and in other species (Choate and Resko, 1992; Clancy *et al.*, 1992; 1994).

Using in vitro binding assays and other techniques, the existence of androgen receptors in the fetal brain and the ontogeny of androgen receptors has been reported in a number of different species (Attardi and Ohno, 1976; Fox *et al.*, 1978; Handa *et al.*, 1988; Toyoka *et al.*, 1989; Vito and Fox, 1982). Since no immunohistochemical data is available on the developmental expression of AR-ir in the brain of other mammalian species, the comparison of the developmental expression of AR-ir across species is not possible at present.

In rodents, several studies have shown that aromatizable androgens are required for both sexual differentiation of the brain during development and control of reproductive functions and behaviors in the adult through their organizational effects (Baum, 1979; Gorski and Jacobson, 1982; Arnold and Schlinger, 1983; Arnold and Gorski, 1984). However, in

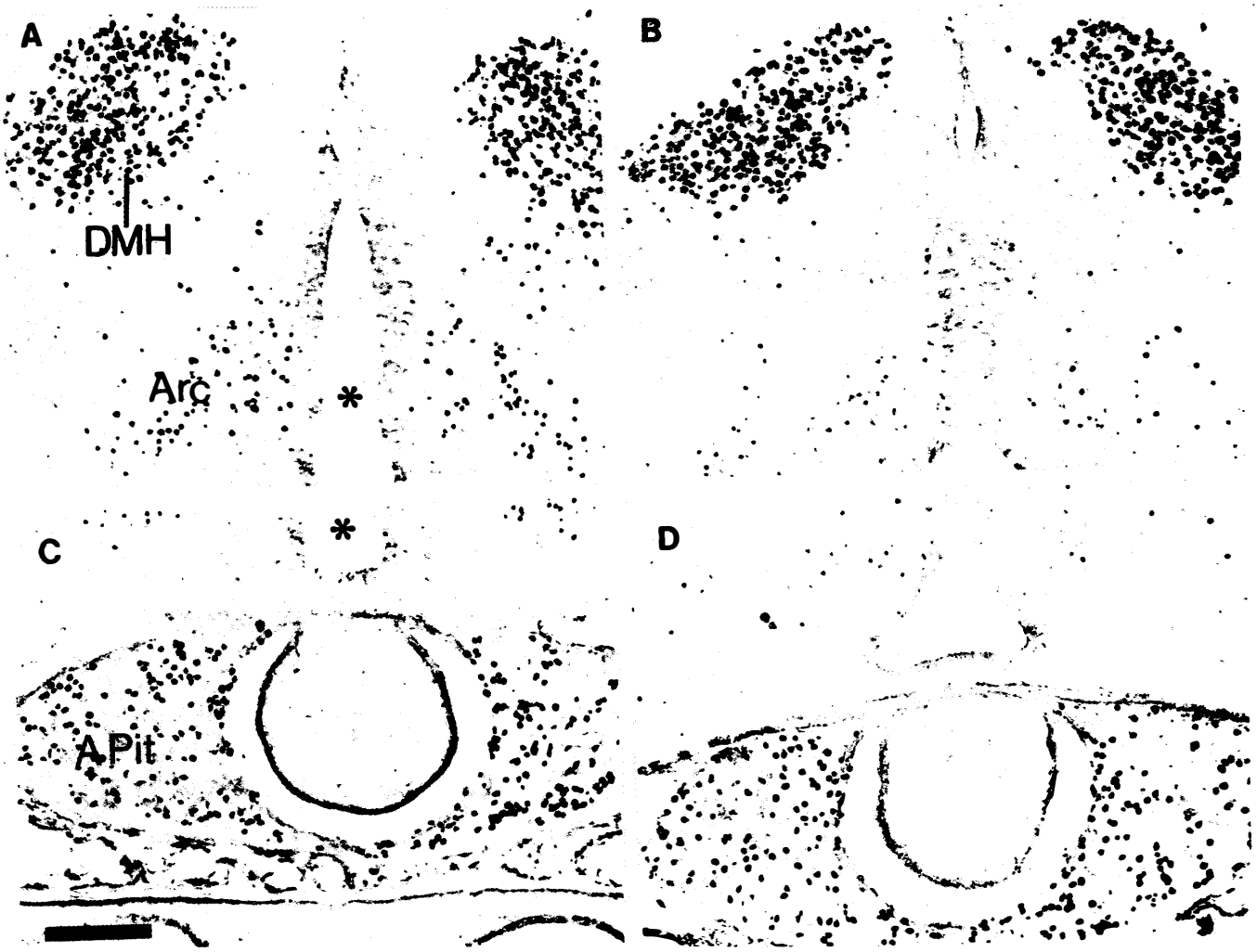

Fig. 7. Photomicrographs demonstrating the expression of androgen receptor-like immunoreactivity in the dorsomedial hypothalamic (A,B; DMH) and arcuate nuclei (A,B; Arc), and anterior pituitary gland (C,D; A.Pit) in the 15 PN male and female after testosterone administration. Testosterone injection at this age resulted in a significant increase in the number of immunoreactive cells and intensity of immunoreactivity both in the male (A,C) and female (B,D). The asterisk indicates the third ventricle. All photomicrographs are at the same magnification. Scale bar= 100 μm.

nonhuman primates and guinea pigs androgens are directly involved in the process of masculinization of the brain (Connolly *et al.*, 1994; Connolly and Resko, 1994). In the neonatal male, testosterone appears to be mainly secreted by the testes, however, the exact source of androgen secretion in perinatal animals is not clear (placental origin or from the fetal gonads and adrenals) (Weisz and Ward, 1980). In the Brazilian opossum testicular tissue is histologically recognizable at birth, and rapidly develops during the first two postnatal weeks with descent into the scrotum between 16 and 20 PN (Fadem *et al.*, 1992; Baker *et al.*, 1993). High androgen levels and aromatase activity have been demonstrated in the opossum pups during the first 2 postnatal weeks (Fadem *et al.*, 1992; 1993). However, it is not clear if the testes are the source of the androgens in the neonatal opossum. Previously, we have reported that, in the opossum estrogen receptor-like immunoreactivity is first detectable at 10 PN and by 15 PN can be detected in many of the areas in which estrogen receptors are present in the adult (Fox *et al.*, 1991b;c). In contrast to that, AR-ir was detected between 10 and 15 PN in the dorsomedial hypothalamic, arcuate, and ventral premammillary

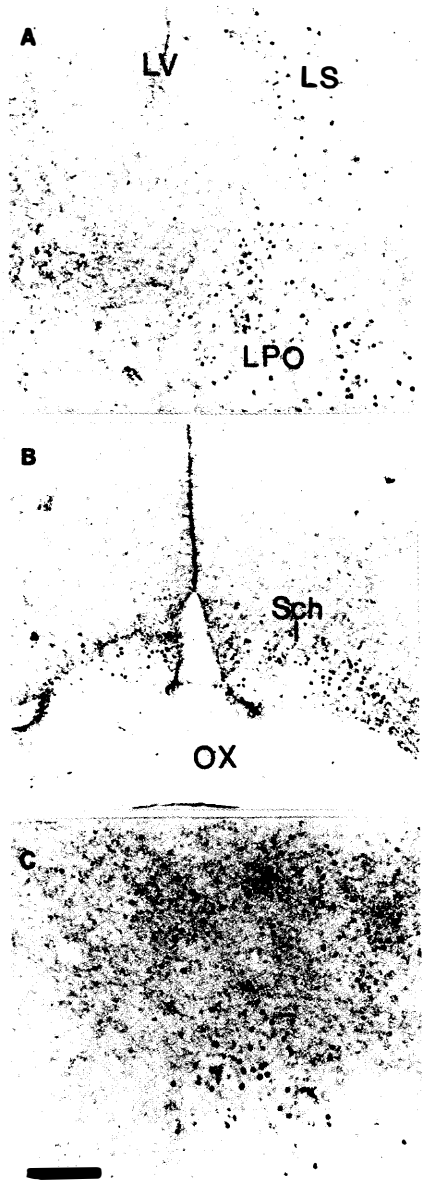

Fig. 8. Testosterone injection at 15 PN resulted in the expression of androgen receptor-like immunoreactivity in several brain areas both in the male and female. Photomicrographs demonstrating androgen receptor-like immunoreactivity in cells in the lateral septum (LS) and lateral preoptic area (LPO; A), suprachiasmatic nucleus (Sch; B), and ventral tegmental area (C) of a 15 PN male. Abbreviations; LV, lateral ventricle; OX, optic chiasm. All photomicrographs are at the same magnification. Scale bar= 100 μm.

nuclei and was not seen in other brain areas of the male until 45 PN. When AR-ir was observed in the ventral nucleus of the lateral septum, between 60 and 80 PN, AR-ir was seen in several of the forebrain and midbrain areas as well.

Androgens are reported to regulate expression of their own receptors in the peripheral tissue and brain (Wood and Newman, 1993; Zhou *et al.*, 1994; Kashon *et al.*, 1995). In order to understand the regulation of androgen receptor expression and the differences in AR-ir in the male and female opossum brain observed during development (results of the present study), we administered testosterone exogenously into developing opossum pups of various ages and killed the animals after 15 minutes or 2 hours postinjection. Testosterone injection 15 minutes prior to tissue collection resulted in no change in AR-ir in the brain of either the male or female, except for an increase in androgen receptor immunostaining in the anterior pituitary gland of both the male and female. Interestingly, testosterone administration 2 hours before tissue collection resulted in the expression of AR-ir in cells in several of the brain areas in the male and female as was seen in males at 60 and 80 PN. Previously, studies using binding assay techniques have reported that exogenous administration of testosterone increased nuclear androgen receptor in the brain of female guinea pig fetuses, whereas male fetuses showed no change in nuclear and cytosolic androgen receptors (Toyoka *et al.*, 1989; Connolly *et al.*, 1994).

At present, we do not know why there is a delay in androgen receptor expression under physiological conditions in the opossum forebrain areas in which AR-ir is detectable after exogenous testosterone administration. Since the opossum does not reach sexual maturity until 5 months of postnatal age (Kuehl-Kovarik *et al.*, 1995), the appearance of androgen receptors in the opossum brain may relate to the onset of puberty, functional maturation of the testis, and/or until adequate androgen plasma levels are achieved, as androgens are reported to regulate androgen receptor immunoreactivity in the brain of prepubertal animals (Kashon *et al.*, 1995). Further, the sex difference in AR-ir in the male and female brain are possibly due to differences in plasma androgen levels. It is also possible that androgen receptor proteins are expressed in low levels before 60 PN, but our technique can not detect these proteins prior to activation through exogenous testosterone administration.

Alternatively, the antibody PG-21, which we used in the present study mainly recognizes the occupied and activated (nuclear) androgen receptor. Previously, it has

also been demonstrated that in the absence of adequate circulating androgens, androgen receptors become cytoplasmic and can be detected using PG-21 antibody and incubation of the tissue in the DAB for 20-60 minutes (Wood and Newman, 1993). In the present study, we observed only nuclear immunostaining even if tissue sections were incubated in the DAB substrate for 40 minutes. However, future studies utilizing measurement of androgen levels during postnatal development of the opossum at appropriate age intervals, experimental manipulation, *in vitro* binding assay, and/or *in situ* hybridization will help understand the exact mechanism of regulation of androgen receptor expression in the opossum brain during development.

Under physiological conditions AR-ir in cells was not detected in the forebrain before 60 PN and in the midbrain and brainstem before 80 PN. However, cells containing AR-ir were present in the dorsomedial hypothalamic, arcuate, and ventral premammillary nuclei between 10 and 15 PN. Presently, the significance of the early expression of AR-ir in cells in these areas of the opossum brain is not known. It is possible that androgen receptor proteins expressed in these areas are more sensitive to even low levels of circulating androgens. Since gonadal steroids are reportedly involved in synaptic organization (Nishizuka, 1992), it is also possible that androgens receptors detected in these nuclei are involved in differentiation of sexually dimorphic neural circuits and brain regions involved in neuroendocrine regulation of gonadotropins and behaviors in the opossum as has been reported for the guinea pig (Connolly and Resko, 1994). However, these results indicate that androgen receptor expression is differentially regulated in different brain regions in the opossum.

One of the mechanisms of hormonal action on reproductive and sexual behaviors is their involvement in the regulation of different neuropeptide systems in the brain. For example, numerous studies have demonstrated that many neuropeptide systems such as arginine vasopressin and cholecystokinin are involved in a number of reproductive and social behaviors (Bloch *et al.*, 1987; 1988; Bluthe and Dentzer, 1993; Wang *et al.*, 1994, Winslow *et al.*, 1993). It has also been reported that expression of these peptides is sexually dimorphic and that their levels of immunoreactivity are influenced by the circulating levels of gonadal steroids (De Vries *et al.*, 1983; 1985; Simerly and Swanson, 1987). We also have observed a sex difference in arginine vasopressin immunoreactivity in the lateral septum of the opossum brain (Iqbal and Jacobson, 1995). This difference becomes apparent by 60 PN and persists into adulthood. We have shown in the present study that AR-ir cells were first detected in the lateral septum at 45 PN and in the bed nucleus of the stria terminalis at 60 PN in the male. Interestingly, no AR-ir cells were seen in the lateral septum of the female of the same age. Thus the findings of the present study suggest that the sex difference in arginine vasopressin immunoreactivity appears in the opossum brain when androgen receptors can be immunohistochemically identified and could potentially be involved in the regulation of arginine vasopressin expression. This concept is supported by findings of Zhou *et al.* (1994), who have reported colocalization of androgen receptors in the vasopressin immunoreactive cells in the rat brain.

In conclusion, we have examined the ontogeny of cells containing AR-ir in the developing opossum brain. Cells containing androgen receptors were first seen in the anterior pituitary at 5 PN and in the brain AR-ir was first detected between 10 and 15 PN. In the 60-80 PN male, AR-ir was seen in all those areas in which AR-ir has been reported for the adult. No AR-ir were detected in the female brain at similar ages, except for moderately immunostained cells in the dorsomedial hypothalamic and ventral premammillary nuclei. Exogenous testosterone administration at 10, 15, and 25 PN resulted in the expression of AR-ir in several of the brain areas both in the male and female as seen in the 60-80 PN male. These findings indicate that the sex difference in the expression of AR-ir in cells is due to differences in plasma androgen levels. The delay of androgen receptor expression in the forebrain of the opossum may be due to inadequate androgen levels in the circulation. Future studies will determine if the "silent" androgen receptors which are not normally seen in the young female have unique functions at this age.

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