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# Distribution of Androgen Receptor Immunoreactivity in Vasopressin- and Oxytocin-Immunoreactive Neurons in the Male Rat Brain*

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**ABSTRACT**

Arginine vasopressin-immunoreactive (AVP-ir) neurons in the bed nucleus of stria terminalis (BST) and medial amygdaloid nucleus are very responsive to gonadal hormones. After gonadectomy, these neurons lose their AVP immunoreactivity and stop expressing AVP mRNA. Testosterone treatment reverses these changes, acting via androgen as well as estrogen receptor-mediated mechanisms. Although AVP-ir neurons contain estrogen receptor immunoreactivity, it is not known whether they also contain androgen receptor immunoreactivity. To answer this question, brains of male rats were stained immunocytochemically for AVP as well as for androgen receptors. In the BST and medial amygdaloid nucleus, respectively, 90.5% and 91.2% of the AVP-ir neurons contained androgen receptor immunoreactivity. In contrast, in the suprachiasmatic nucleus, the supraoptic nucleus, and the magnocellular portion of the paraventricular nucleus (PVN), none of the AVP-ir neurons contained androgen receptor immunoreactivity. In the ventral zone of the medial parvocellular part of the PVN (mpvPVN), 4.3% of the scattered AVP-ir neurons contained androgen receptor immunoreactivity. One of the control experiments, *i.e.* staining sections for oxytocin (OT) rather than AVP, revealed that although OT-ir neurons in the supraoptic and magnocellular portion of the PVN did not contain androgen receptor immunoreactivity, 52.5% of the OT-ir neurons in the mpvPVN did. The results suggest that androgens can bind to androgen receptors in AVP-ir neurons in the BST and medial amygdaloid nucleus, possibly to influence AVP expression. The results also suggest that androgens can bind to androgen receptors in AVP-ir and OT-ir neurons in the mpvPVN. The function of the latter interaction, however, is unclear. (*Endocrinology* **134:** 2622–2627, 1994)

ARGININE vasopressin-immunoreactive (AVP-ir) neurons in the bed nucleus of the stria terminalis (BST) and medial amygdaloid nucleus in rat brains are very responsive to gonadal hormones. After gonadectomy, these neurons lose their AVP immunoreactivity and stop expressing AVP mRNA (1–4). Testosterone treatment of gonadectomized male rats prevents these changes (1–3, 5, 6).

In the brain, testosterone can bind to estrogen receptors after aromatization to estradiol (7). Alternatively, testosterone can bind to androgen receptors either directly or after reduction to 5$\alpha$-dihydrotestosterone (DHT) (8). Therefore, testosterone can potentially influence AVP gene expression in BST and medial amygdaloid nucleus neurons through mechanisms that are mediated by estrogen as well as androgen receptors. Indeed, estradiol treatment partly restores the AVP immunoreactivity and AVP mRNA levels in the BST and medial amygdaloid nucleus of castrated male rats (5, 6), whereas a combination of DHT and estradiol treatment fully restores AVP immunoreactivity and AVP mRNA levels (5, 6).

Although these data suggest that estrogen receptors as well as androgen receptors are involved in the effects of testosterone on AVP production, it is not clear where these steroid receptors are located. A previous study suggests that the estrogen receptors that mediate the effects of testosterone on AVP expression may be located in AVP-producing neurons themselves, as almost all AVP-ir neurons in the BST and medial amygdaloid nucleus also contain estrogen receptor immunoreactivity (9). However, it is not yet known whether the same is true for androgen receptors. The present study mapped the distribution of AVP and androgen receptor immunoreactivity and tested whether AVP-ir neurons contained androgen receptor immunoreactivity. Although this study was focussed on neurons in the medial amygdaloid nucleus and BST, it also considered neurons in the suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN), and supraoptic nucleus (SON), in which AVP production continues after castration. To assess the possibility of cross-reactivity of the AVP antiserum with oxytocin (OT) in the PVN, the present study also tested whether OT-ir neurons contained androgen receptor immunoreactivity.

## Materials and Methods

Male Long-Evans rats (260–280 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and maintained under a 14-h light, 10-h light cycle, with food and water freely available. Ten gonadally intact rats were used to map vasopressin and androgen receptor immunoreactivity. Without colchicine treatment, only a fraction of the vasopressin-immunoreactive cells is visible in the BST and medial amygdaloid nucleus. Therefore, to increase the intensity of AVP immunostaining in the BST and medial amygdaloid nucleus, 30 $\mu$g colchicine in 15 $\mu$l 0.15 M NaCl were injected stereotaxically into the right lateral ventricle of six of these rats under chloral hydrate-pentobarbital anesthesia (containing 0.25 M chloral hydrate, 0.08 M magnesium sulfate, 4.5 mM

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pentobarbital, 3.0 M ethyl alcohol, and 4.5 M propylene glycol; 2.8 ml/kg BW, ip) 24 h before perfusion. As AVP-ir neurons in other areas tend to be overstained after colchicine treatment, the remaining four rats were perfused without colchicine pretreatment. Colocalization of AVP and androgen receptor immunoreactivity in the BST and MA was studied in the sections from colchicine-treated rats. Colocalization in other areas was studied in noncolchicine-treated rats. Six other rats were used as controls for the androgen receptor immunoreactivity. These rats were gonadectomized under chloral hydrate-pentobarbital anesthesia. Four days later, two of them were injected ip with testosterone (Sigma Chemical Co., St. Louis, MO; 100 μg/100 g BW), two others with DHT (Sigma; 100 μg/100 g BW), and the remaining two with vehicle only (1.0 ml sesame oil), 15 min before death in each case.

All rats were deeply anesthetized and perfused through the ascending aorta with 100 ml 0.15 M NaCl, followed by 4% acrolein, 0.25% glutaraldehyde, 0.75% polyvinylpyrrolidone (PVP; average mol wt, 40,000; Sigma) in 0.1 M sodium phosphate buffer, pH 7.4, for 10 min (~400 ml/animal). The brains were removed and postfixed at 4 C in 4% acrolein and 0.75% PVP in 0.1 M PBS for 12 h.

Fifty-micron transverse sections cut with a vibratome were collected in four vials containing 0.05 M Tris buffer (Sigma) and 0.15 M NaCl with 1.5% PVP, pH 7.6 (PVP-TBS), so that each vial contained one of every four sections. For each of the intact animals, one of these vials was used for androgen receptor immunocytochemistry only, and another vial was used for AVP as well as androgen receptor immunocytochemistry. The remaining vials (including the sections of castrated animals) were used for specificity tests.

To stain for androgen receptor immunoreactivity, free-floating sections were incubated with the following solutions at room temperature: 1) PVP-TBS, three times for 5 min each time; 2) 0.05% $NaBH_4$ in PVP-TBS for 10 min; 3) PVP-TBS, three times for 5 min each time; 4) rabbit antiandrogen receptor immunoglobulin G (PG21; gift from Geoffrey Greene, University of Chicago, Chicago, IL), 0.47 μg/ml (diluted 1:2000) in 0.5% Triton X-100 in TBS (Tris-Triton) with 0.1% gelatin and 2% normal goat serum for 12 h; 5) Tris-Triton, three times for 5 min each time; 6) biotinylated goat antirabbit immunoglobulin G (Vector Laboratories, Burlingame, CA) diluted in Tris-Triton with 2% normal goat serum for 1.5 h; 7) Tris-Triton for 5 min and TBS, twice for 5 min each time; 8) Vectastain Elite ABC reagent prepared according to the instructions of the Elite ABC Kit solution (Vector Laboratories) for 45 min; 9) TBS, three times for 5 min each time. After these steps, sections were stained by incubation in TBS containing 0.05% 3–3′-diaminobenzidine (DAB), 0.4% ammonium chloride, 0.02% glucose oxidase, 1.5 mM nickel-ammonium sulfate, and 0.3% β-D-glucose for about 20 minutes, which produced a dark blue staining of the nuclei of specific neurons (glucose oxidase-nickel-DAB) (modified from Ref. 10). To stain for AVP immunoreactivity as well, the sections were incubated with rabbit anti-AVP (ICN Immunobiologicals, Lisle, IL), diluted 1:4000 in Tris-Triton with 2% normal goat serum, at 37.5 C for 45 min. The sections were then incubated at room temperature in solutions 5–9 described above for androgen receptor staining. After these steps, sections were stained with 0.05% DAB in TBS with 0.002% $H_2O_2$ for about 20 min, which produced a light brown staining of the cytoplasm in specific neurons. After staining, the sections were mounted, air dried, and coverslipped. Sections through the entire bed nucleus of the stria terminalis, hypothalamus, and medial amygdaloid nucleus were inspected with a Zeiss Axioscope (Zeiss, New York, NY) under brightfield illumination. To identify brain areas for camera lucida drawings, sections were also inspected under darkfield illumination.

To test the specificity of the androgen receptor staining, sections were incubated with PG21 serum that had been treated with a 10-fold molar excess of the peptide AR21, which corresponds to the 21-amino acid sequence at the N-terminal end of the rat androgen receptor protein that had been used to raise the antiserum (11). In addition, sections from male rats that had been castrated 4 days earlier and treated with oil, testosterone, or DHT 15 min before perfusion were stained for androgen receptor immunoreactivity. Finally, sections containing the CA1 field of the hippocampus, which expresses androgen receptor mRNA, and the lateral habenular nucleus, which does not express androgen receptor mRNA (12), were inspected as an additional control on the specificity.

To test for specificity of the AVP staining, sections were stained for androgen receptors as described above and subsequently incubated with AVP antiserum that had been treated with 50 μM AVP (Sigma). As the AVP antiserum could cross-react with OT in the PVN and SON, sections were also incubated with AVP antiserum that had been treated with 50 μM OT (Sigma). In addition, sections from four noncolchicine-treated animals were immunostained for androgen receptor as well as OT using rabbit anti-OT antiserum VA10 (gift from Dr. Harold Gainer, NIMH, Bethesda, MD), which does not cross-react with AVP (13). VA10 was diluted 1:4000 in Tris-Triton with 2% normal goat serum. The rest of the protocol was similar to that used for AVP staining.

## Results

In intact male rats, the androgen receptor immunostaining protocol that we used resulted in a strong and clear dark blue staining in cell nuclei in specific areas, without any staining in the cytoplasm (Fig. 1, A and C). This nuclear immunostaining was absent in sections stained with PG21 that had been treated with the AR21 peptide. Nuclear immunostaining was also absent in castrated rats treated with vehicle, but was present in castrated rats that had been treated with DHT or testosterone 15 min before perfusion (Fig. 2). No nuclear immunostaining for androgen receptor was found in the lateral habenular nucleus, whereas it was found in the CA1 area of hippocampus (Fig. 3). Colchicine-

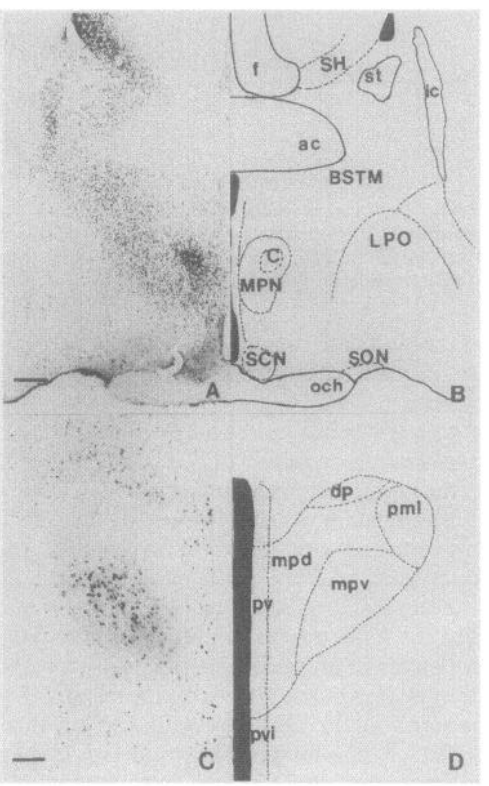


FIG. 1. Distribution of cell nuclei with androgen receptor immunoreactivity in sections containing areas that produce AVP or OX (*left panels*) and mirror-imaged camera lucida drawings of the same sections (*right panels*). Nuclear androgen receptor immunoreactivity was found in the BST and mpvPVN. Almost no nuclear immunostaining could be found in SCN, SON, and other parts of the PVN. ac, Anterior commissure; dp, dorsal parvocellular part of PVN; MPN, medial preoptic nucleus; C, central part of MPN; mpd, dorsal zone of the medial parvocellular part of PVN; pml, lateral zone of the posterior magnocellular part of PVN; och, optic chiasm. *Bar* = 30 μm in A and 15 μm in C.

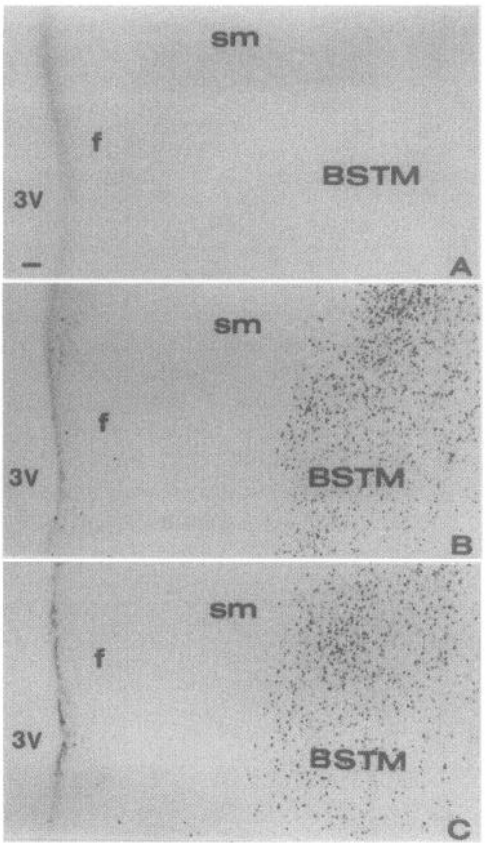


FIG. 2. Nuclear androgen receptor immunostaining in a castrated rat (A) and a castrated rat treated with DHT (B) or with testosterone (C). 3V, Third ventricle; BSTM, bed nucleus of the stria terminalis; f, fornix; sm, stria medullaris thalamus. *Bar* = 30 μm.

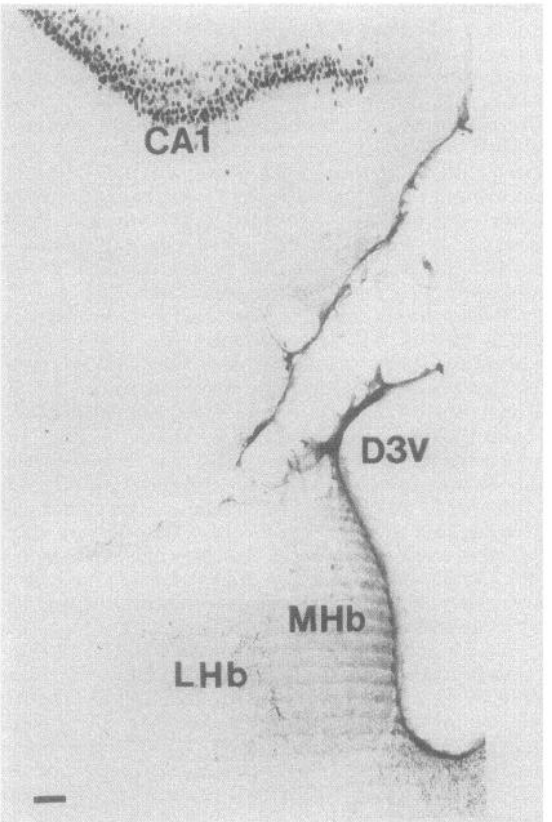


FIG. 3. Distribution of cell nuclei with androgen receptor immunoreactivity at the level of the lateral habenular nucleus (LHb) and the CA1 field of the hippocampus. MHb, Medial habenular nucleus; D3V, dorsal part of third ventricle. *Bar* = 30 μm.

treated rats showed the same distribution of androgen receptor-immunoreactive neurons as rats that were not treated with colchicine.

AVP-ir neurons could be found in the SCN, SON, PVN, BST, and medial amygdaloid nucleus and in small clusters scattered through the hypothalamus; nuclear androgen receptor immunostaining was found in the BST, medial amygdaloid nucleus, and the ventral zone of the medial parvocellular part of PVN (mpvPVN; Fig. 1). Almost no nuclear immunostaining could be found in the SCN, SON, and parts of the PVN other than the mpvPVN (Fig. 1).

In sections stained immunocytochemically for AVP as well as for androgen receptors, 90.5% of a total of 559 inspected AVP-ir neurons in the BST (recognizable by brown cytoplasm) and 91.2% of a total of 431 inspected AVP-ir neurons in the medial amygdaloid nucleus contained dark blue nuclear immunostaining, indicating androgen receptor immunoreactivity (Fig. 4). AVP-ir neurons in the SON, SCN, and the small hypothalamic clusters, however, contained no nuclear immunostaining. Most of the AVP-ir neurons in PVN also did not contain nuclear immunostaining. However, nuclear androgen receptor immunostaining was found inside some of the AVP-ir neurons in the mpvPVN.

Pretreating the AVP antiserum with AVP blocked immunostaining of all neurons in the BST, medial amygdaloid nucleus and SCN. It also dramatically reduced AVP immunostaining in the PVN and SON, leaving only ghost-like cells. Pretreating the AVP antiserum with OT reduced the number of immunostained neurons in the mpvPVN; 4.3% of the 187 inspected remaining AVP-ir neurons contained androgen receptor immunoreactivity. In sections stained immunocytochemically for OT as well as for androgen receptors, 52.5% out of 461 inspected OT-ir neurons in the mpvPVN contained androgen receptor immunoreactivity (Fig. 5). No dark blue nuclear immunostaining was found in OT-ir neurons in other parts of PVN or in any OT-ir neuron in the SON, anterior commissural nucleus, or OT-ir neurons present in small hypothalamic clusters.

## Discussion

The AVP-ir neurons in the BST and medial amygdaloid nucleus are extremely responsive to gonadal hormones. After castration, these neurons can no longer be stained immunocytochemically for AVP or be labeled for AVP mRNA (1, 4–6). Testosterone treatments prevent these changes, presumably by androgen receptor- as well as estrogen receptor-mediated mechanisms (5, 6). A previous study had already demonstrated that virtually all AVP-ir neurons in the BST and medial amygdaloid nucleus contain estrogen receptor immunoreactivity (9). The present study showed that vir-

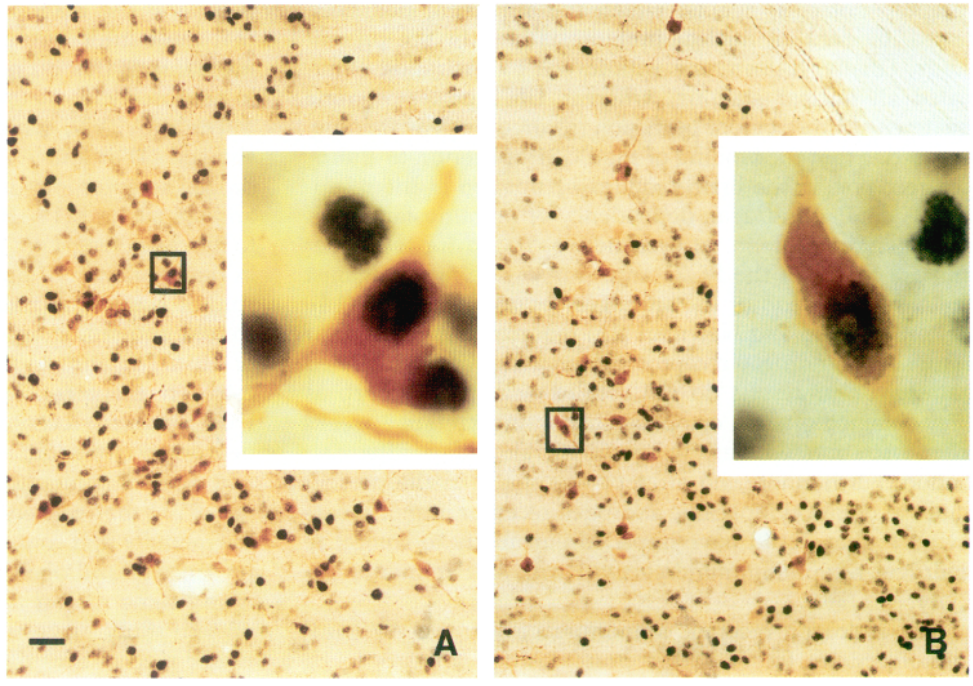


FIG. 4. The colocalization of androgen receptor and AVP immunoreactivity in neurons in the BST (A) and the medial amygdaloid nucleus (B). The *insets* in A and B show magnifications of individual AVP-ir neurons that contain androgen receptor immunoreactivity. *Bar* = 40 μm.

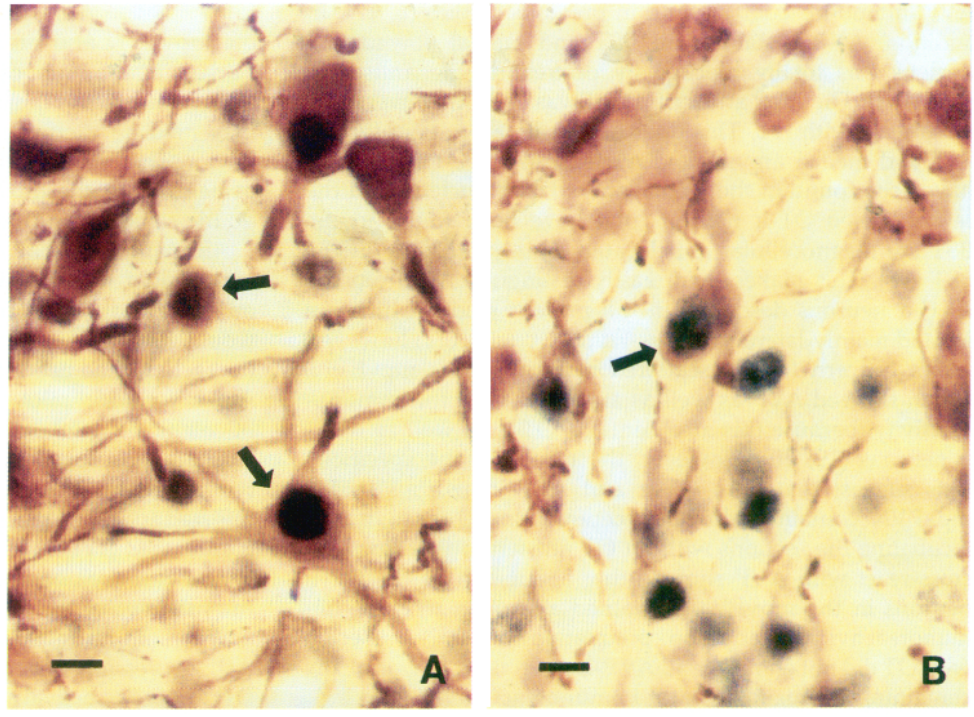


FIG. 5. Distribution of androgen receptor immunoreactivity in the mpvPVN in consecutive sections that were also stained for OT (A) or AVP (B) immunoreactivity. *Arrows* indicate OT- and AVP-immunoreactive neurons that also contain androgen receptor immunoreactivity. *Bar* = 10 μm.

tually all of these AVP-ir neurons also contain androgen receptor immunoreactivity. The data from the previous and present study combined suggest that estrogens as well as androgens may influence AVP production by acting directly on AVP-producing neurons in the BST and medial amygdaloid nucleus.

The androgen receptor immunoreactivity found in the present study appears to be specific, as its distribution closely resembled the distribution of androgen receptors indicated by autoradiography, *in situ* hybridization, and other immunocytochemistry studies (8, 12, 14). The absence of androgen receptor immunostaining in the lateral habenular nucleus, which contains an abundance of estradiol-concentrating cells and estrogen receptor mRNA (12, 15), and the presence of androgen receptor immunostaining in the CA1 area of the hippocampus, which contains very few estradiol-concentrating cells and hardly any estrogen receptor mRNA (13, 15), also suggest that the antiserum recognizes androgen receptors, but not estrogen receptors. In addition, pretreating the antiserum with the AR21 peptide fragment of the androgen

receptor molecule, against which the antiserum had been raised, blocked all nuclear immunostaining in the present study. Finally, testosterone withdrawal by castration eliminated all nuclear staining, whereas DHT or testosterone treatment 15 min before perfusion restored nuclear staining, suggesting that the binding of the PG21 antiserum to androgen receptors in the brain requires occupation of the receptors. A similar androgen dependency of immunostaining of androgen receptors in rat prostate was found for two other antisera that, like the PG21 antiserum, were raised against the N-terminal end of the androgen receptor molecule (14). Recently, Wood and Newman (16) reported the presence of cytoplasmic androgen receptor immunoreactivity in castrated hamsters. Our results do not conflict with their report, as they obtained optimal cytoplasmic immunostaining 2 weeks after castration, whereas we perfused rats 4 days after castration. Furthermore, no attempt was made in the present study to obtain cytoplasmic androgen receptor immunoreactivity, as this would have impeded our ability to colocalize androgen receptor immunoreactivity with AVP immunoreactivity.

Like androgen receptor immunoreactivity, the AVP immunoreactivity found in the present study appears to be specific. Its distribution closely resembles the distribution of AVP immunoreactivity found in previous studies (6, 17). In addition, AVP-ir staining in BST and medial amygdaloid nucleus was totally blocked by pretreating the antiserum with AVP. The AVP antiserum that was used, however, may cross-react with OT. This does not create a problem for the BST and medial amygdaloid nucleus, as no OT-ir neurons have been found in the medial amygdaloid nucleus, and the few scattered OT-ir neurons that have been found in the BST are magnocellular and located mainly medially in the BST (18). This makes them easily distinguishable from the AVP-ir neurons described in the present study, which are located mainly laterally. Cross-reactivity with OT, however, was a concern for the scattered neurons in the mpvPVN, which showed AVP as well as androgen receptor immunoreactivity, because this area contains OT-ir as well as AVP-ir neurons (19, 20). However, the results of the specificity tests suggest that both OT-ir and AVP-ir neurons in the mpvPVN contain androgen receptor immunoreactivity, as incubating the sections with an OT antiserum that does not cross-react with AVP showed that about half of the OT-ir neurons in the mpvPVN contained androgen receptor immunoreactivity. In addition, pretreating the AVP antiserum with OT did not eliminate all AVP-ir neurons in the mpvPVN containing androgen receptor immunoreactivity.

AVP-ir neurons in the SCN did not show androgen receptor immunoreactivity. There are, indeed, no reports of androgen effects on AVP synthesis in the SCN. In addition, magnocellular AVP-ir neurons in the PVN and SON, which project to the neurohypophysis (19), also showed no androgen receptor immunoreactivity. Androgens appear to influence AVP neurons in the SON and PVN, however, because they inhibit AVP neurosecretion, although it is not known whether this effect involves changes in AVP synthesis (21). The absence of androgen receptor immunoreactivity in the SON and the magnocellular portion of the PVN suggests that these inhibitory actions of androgens are mediated by mechanisms that do not involve nuclear androgen receptors in neurosecretory neurons of the PVN and SON.

The mpvPVN, which is the only area in the PVN that showed OT-ir and AVP-ir neurons that also contained androgen receptor immunoreactivity, projects to the hind brain and spinal cord (19, 20). As many of these neurons project to the dorsal vagal complex, they may play an important role in the regulation of autonomic mechanisms (19, 20). Earlier studies combining neurophysin immunocytochemistry and steroid autoradiography had suggested that more than half of all OT-ir and AVP-ir neurons in the mpvPVN contain estrogen receptors (22), although this could not be confirmed with estrogen receptor immunocytochemistry for AVP-ir neurons (9). In addition, a combination of retrograde tracing and steroid autoradiography showed that neurons in the mpvPVN that concentrated estradiol project to the area in the medulla that contains the dorsal vagal complex (23). The presence of androgen receptor immunoreactivity in the OT-ir and some AVP-ir neurons in this same area found in the present study suggests that androgens might influence autonomic mechanisms by acting directly on descending projections of the mpvPVN as well. Recently, Wagner *et al.* (24) found that in male rats, estradiol-concentrating neurons in the mpvPVN also project to the sexually dimorphic spinal nucleus of the bulbocavernosus. As this nucleus innervates striated muscles at the base of the penis, mpvPVN projections may mediate the influence of estradiol on male sexual behavior. The presence of androgen receptor immunoreactivity in neurons in the mpvPVN suggests that these projections do the same for androgen.

Although almost all AVP-ir neurons in the BST and medial amygdaloid nucleus also contained androgen receptor immunoreactivity, it is not clear whether those that did not would have shown androgen receptor immunoreactivity if the sections had been stained under more optimal conditions. As we found in pilot experiments that either larger dosages of colchicine (>50 $\mu$g) or longer exposure to colchicine (>48 h) than those used in this study attenuated the androgen receptor staining, we used a dosage and exposure time that affected androgen receptor staining only marginally, whereas it enhanced AVP staining considerably.

The presence of androgen receptor immunoreactivity in the majority of the AVP-ir neurons in the BST and medial amygdaloid nucleus suggests that androgens may influence AVP expression by acting directly on these neurons. By itself, DHT does not activate AVP expression in the BST and medial amygdaloid nucleus of gonadectomized male rats, but when given in combination with estradiol, DHT increases the density of AVP-ir projections in the septum and AVP mRNA levels in the BST (5, 6). As virtually all AVP-ir neurons in the BST and medial amygdaloid nucleus also contain estrogen receptor immunoreactivity (9), the synergistic effect of DHT and estradiol on the expression of AVP in those neurons could be mediated by androgen and estrogen receptor-related mechanisms within those neurons. So far, however, the mechanisms by which androgens and estrogens influence

AVP gene expression remain unknown. Synergistic effects of androgens and estrogens have been found in other cases, for example, the stimulation of male sexual behavior (25, 26), aggressive behavior (27), the development of sexually dimorphic nuclei in songbirds (28), and even in the effect of gonadal hormone replacement therapy on changes in affect in surgically menopausal women (29). Given the clarity of the effects of gonadal steroids on AVP gene expression, the AVP neurons of the BST and medial amygdaloid nucleus may provide a good model to study cellular mechanisms underlying synergistic actions of gonadal steroids.

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