

Distribution of Androgen Receptor mRNA Expression and Immunoreactivity in the Brain of the Green Anole Lizard

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Abstract

Male courtship and copulation are androgen dependent in the green anole lizard, and female receptivity can be facilitated by testosterone. However, only a few, and relatively large, regions in the brain have been implicated in the control of these behaviours. *In situ* hybridization and immunohistochemistry were therefore used to determine in detail where androgens are likely to act in the brains of breeding males and females. A 697-bp fragment of the anole androgen receptor (AR) was cloned from total RNA isolated from the kidney, which contains the highly androgen-sensitive renal sex segment. The cloned fragment spanned part of the C, the entire D, and part of the E domains, and shared a high degree of similarity with the AR of various species. ³⁵S-labelled antisense and sense probes were generated from the 697-bp fragment for use in *in situ* hybridization, and the AR antibody PG-21 was used for immunohistochemistry. Both sexes consistently had AR mRNA expression and immunoreactivity in areas associated with vertebrate reproductive behaviours and in motor areas of the brainstem. Interestingly, the PG-21 antibody produced labelling in both the nucleus and cytoplasm, including neuronal processes. The distribution of mRNA and immunoreactivity were comparable in males and females, and the amount of labelling was generally similar, although slightly greater in females. The expression pattern of AR in this species supports the idea that distribution is highly conserved among vertebrates, but that it probably does not dictate behavioural differences between the sexes in anoles.

Androgens activate sexual behaviours in the green anole lizard (*Anolis carolinensis*) (1–3). Although testosterone is the most effective, its metabolite dihydrotestosterone can also facilitate masculine courtship display and copulation (3, 4), and inhibiting 5 α -reductase activity (which synthesizes dihydrotestosterone from testosterone) reduces courtship behaviour (5). In contrast, systemic administration of oestradiol does not stimulate masculine behaviours (1, 2, 6), and blocking aromatase activity (which converts testosterone to oestradiol) has no effect on testosterone-induced courtship or copulatory behaviour (6). In females, testosterone also facilitates receptivity (3). Unlike males, however, the effect on one measure of receptivity (latency to copulation) is attenuated by concurrent administration of an aromatase inhibitor (6), suggesting that the

conversion of testosterone to oestradiol contributes to the regulation of feminine behaviours.

Lesion and implant studies in anoles suggest that steroid hormones act on the anterior hypothalamus-preoptic area (AH-POA) and basal hypothalamus to facilitate reproductive behaviours (4, 7–9). However, it is difficult, from these experiments, to determine more precisely the critical regions within the AH-POA that regulate the expression of these behaviours. Tract tracing experiments (10, 11) also implicate neurones in the ventral motor nucleus of the facial nerve and in the glossopharyngeal and vagal portions of nucleus ambiguus (AmbIX/VIIImv and AmbX) in extension of the dewlap, a throat fan displayed during courtship and aggressive interactions (12). An autoradiography study (13) has confirmed the

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presence of androgen binding in the POA and hypothalamus, as well as other limbic, and some brainstem areas that are more rostral than those involved in dewlap extension. However, the tissue in that study was taken during the nonbreeding season, and the animals had been castrated for a month before the administration of tritiated-hormone. Because androgens can up-regulate the androgen receptor (AR) in many tissues (reviewed in (14)), including the brain (15–18), it is possible that the binding detected in the study was an underestimation of the population of receptors available to reproductively active individuals. The purpose of the present study was to determine the distribution of AR more completely and under more physiological conditions by localizing both its mRNA and presumptive protein (AR-like immunoreactivity; AR-ir) in the brains of intact males and females during the breeding season.

Materials and methods

Animals

Male and female green anole lizards (*A. carolinensis*) were purchased from Buck's or Fluker Farms (Port Allen and La Place, Louisiana, USA) during the spring and summer breeding season. The animals were housed in the laboratory in groups consisting of one male and at least two females. To maintain reproductive activity, the lizards were exposed to a long day photoperiod (14L:10D), and a heat lamp was placed over each cage. Lizards were fed crickets or meal worms three times a week, and allowed to acclimatise to laboratory conditions for at least 2 weeks prior to tissue collection. Reproductive condition was confirmed by inspection of the gonads under a dissecting microscope. Males had medium to large testes and hypertrophied vasa deferentia, and females had at least one yolking follicle. All procedures adhered to guidelines of the Michigan State University Animal Use and Care Committee and the National Institutes of Health.

Cloning and sequencing of partial AR mRNA sequence

Two breeding males were rapidly decapitated and total RNA from the kidneys was isolated using Trizol (Gibco Life Technologies Rockville, MD, USA) according to manufacturer's instructions. The kidney was selected because it contains the renal sex segment (2, 19), a region that performs a function similar to the mammalian prostate (20), and, like the prostate, is highly androgen sensitive. The kidney has also been used to successfully clone the AR from another lizard species (21). Five micrograms of total RNA was incubated for 10 min at 70 °C with 500 nm oligo dT primer and reverse transcribed with SuperScript II reverse transcriptase (Gibco Life Technologies). One tenth of the reverse transcription reaction was used in the subsequent PCR reactions. Primers were identified from highly conserved regions within a consensus sequence derived by a multiple sequence alignment of the ARs from 10 different species (restriction enzyme sites are underlined and parentheses designate differences in degenerate primers): forward primer – CAAATCTAGAGGATCCCTTCAA(A/G)AGAGCCGCTGAAGGGAAA-CAG (last 28 bases correspond to human AR nucleotides 1746–1773); reverse primer – CAAAGCGGCCGCTCGAGGCTGAAGAG(A/G/C/T)AGC-AG(A/G/C/T)GC(C/T)TTCATGCACAGG (last 30 bases correspond to human AR nucleotides 2412–2442). In addition to the template, each PCR reaction contained 20 µM Tris-HCl (pH 8.4), 50 mM KCl, 3 mM MgCl₂, 200 nM of each primer, 200 mM dNTPs (Gibco Life Technologies) and 2.5 units of Taq polymerase (Roche/Boehringer Mannheim, Indianapolis, IN, USA). Samples were incubated at 95 °C for 5 min and amplified for 35 cycles. Each cycle consisted of 45 s denaturation at 95 °C, 45 s annealing at 60 °C and 1 min elongation at 72 °C. The resulting fragment was digested with BamHI and XhoI (Roche/Boehringer Mannheim), cloned into a pSG5 based cloning vector, pTL1, and sequenced using the dye terminator technique on an ABI/Prism automated sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA). Sequence analysis was performed using MacVector 6.5 and the GCG Wisconsin Package (Accelrys, San Diego, CA, USA). For use as a probe in *in situ* hybridization, the cDNA was re-cloned into pBluescript and the sequence was confirmed in both directions with the same technique.

In situ hybridization

The distribution of AR mRNA was examined in the brains from five males and six females. The animals were rapidly decapitated and brains and kidneys (from males only) were removed and immediately frozen in isopentane cooled with dry ice. Tissue was stored at –80 °C until sectioning. Frozen coronal sections were cut at 20 µm, thaw-mounted onto Superfrost Plus slides (Fisher Chicago, IL, USA), and stored with desiccant at –80 °C. Adjacent sections were placed on separate slides so that different probes (antisense and sense) could be used on the same brain. ³⁵S-labelled (UTP, specific activity 1250 Ci/mmol; Perkin Elmer Life Sciences, Boston, MA, USA) antisense and sense probes were transcribed using T7 (antisense) and T3 (sense) polymerases and nucleotides per manufacturer's instructions (Stratagene RNA Transcription Kit, Stratagene, LaJolla, CA, USA), except incubation time was increased to 60 min. The cDNA template was digested with RNase-free Dnase (1 U/µL; Stratagene) for 15 min at 37 °C. To remove unincorporated nucleotides, the solution was diluted 1:8 with NETS (150 mM NaCl, 10 mM EDTA, 50 mM Tris, 0.1% SDS, pH 7.5) and dithiothreitol (DTT, 20 mM final concentration) and spun through a 1-mL G50 Sephadex column.

Before hybridization, sectioned tissue was briefly thawed to room temperature, fixed for 5 min in 4% formalin in phosphate buffered saline (PBS; pH 7.0), rinsed for 2 min in 2 × SSC, and treated for 10 min with 0.25% HCl in 1 × triethanolamine buffer (0.1 M triethanolamine, 0.9% NaCl). The slides were then rinsed for 2 min in 2 × SSC, dehydrated through a graded alcohol series, delipidated in chloroform, rinsed in 95% ethanol, and air dried. The purified probe was heat denatured for 60 s at 100 °C and diluted to 2 × 10⁷ CPM/mL with hybridization buffer (50% formamide, 10% dextran sulphate, 0.3 M NaCl₂, 0.01 M Tris, 1 mM EDTA, 1 × Denhardt's solution, 50 mM DTT, 0.05 mg/mL yeast tRNA). The diluted probe was placed on the sections (1 × 10⁶ cpm/slide), and allowed to hybridize for 20–24 h at 55 °C. The sections were rinsed twice in 2 × SSC, RNase digested (50 µg/mL in 2 × SSC) for 50 min at 37 °C, and subjected to the following stringency washes at 55 °C, all of which included 0.1% β-mercaptoethanol: 1 × SSC for 15 min (twice), 50% formamide in 1 × SSC for 30 min, and 0.1 × SSC for 15 min (twice). Finally, the slides were dipped in Kodak NTB-2 autoradiographic emulsion, air dried and exposed for 4 weeks at 4 °C. After developing with Kodak Dektol developer, the sections were lightly counterstained with thionin or haematoxylin and eosin, dehydrated, cleared in xylene and coverslipped. Sections of male kidney and brains from both sexes were processed simultaneously. The two anatomical stains allowed equivalent detection of cell morphology in the brain.

The distribution of cells labelled with silver grains was visualized under both bright- and dark-field, using an Olympus BX-60 light microscope (e.g. Figs 1A and B). The specificity of the antisense probe was indicated by extensive labelling of the renal sex segment and epididymis, which was absent in sections exposed to the sense probe (Fig. 2). Initially, neurones were considered specifically labelled by the antisense probe if silver grains were densely clustered over neurone cell bodies, and similar clustering did not occur in the same regions in alternate sections exposed to the sense probe (Figs 1A and C). The distribution of cells in the brain was determined for all individuals based on neuroanatomical maps for lizards (22–25), and was highly consistent across animals.

Specificity of labelling in the brain was confirmed from one representative individual of each sex. Because the silver grains were too densely clustered in some areas to accurately count by visual inspection, Scion Image (NIH image software) was used to assess the percent area of the neuronal soma covered by silver grains (see (26) for a similar method). In each region that appeared to contain specific labelling, the area of the soma was measured in 20 randomly selected cells (10 on each side of the brain). Then the soma area covered by silver grains was determined for each cell using the density slice function. From these two measures, the percent soma area covered by silver grains was calculated. To determine nonspecific binding in the tissue, this technique was used in every brain region thought to contain labelling (see Fig. 3) on adjacent sections exposed to the sense probe. In addition, the same process was used to analyse silver grains in nucleus rotundus exposed to the antisense probe. Based on autoradiography (13) and immunohistochemistry (see below), this area does not appear to contain AR. Background levels were equivalent in sense-treated sections and nucleus rotundus (data not shown). Thus, individual cells were considered to be specifically labelled if the percent soma area covered by silver grains was at least 5 times the average measurement for the adjacent sense-treated section. By this criterion, a minimum of 20% of cells was labelled in each region reported in the Results section. For most areas, however, more than half of the analysed cells were specifically labelled.

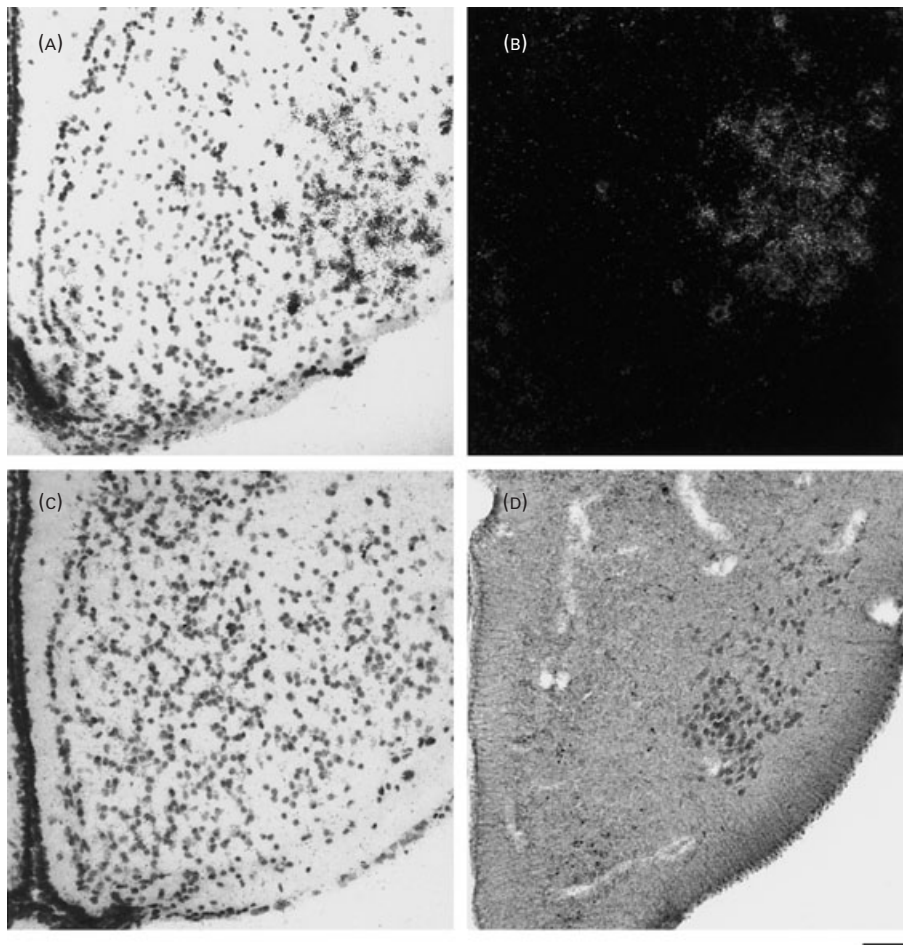


FIG. 1. AR expression in the ventromedial hypothalamic nucleus. Specific labelling of mRNA was observed in sections exposed to the antisense probe (A and B, bright- and dark-field, respectively), but not in adjacent sections exposed to the sense probe (C). Cellular nuclei containing AR immunoreactivity occurred in the same region of the ventromedial hypothalamic nucleus (D). Photographs in this and subsequent figures (2, 4, 7), were taken with a Kodak DCS 410 digital camera using Adobe PhotoShop on a Macintosh Power PC. Scale bar = 50 μ m.

The same method was then used on each brain to quantify levels of AR expression in areas potentially related to anole reproductive behaviours: (i) a particularly dense region of labelling in the posterior POA (POAp; identified with a medial box in Fig. 3D), (ii) the medial preoptic area (MPOA; Fig. 3B), (iii) the ventromedial nucleus of the amygdala (VMN-amy; Fig. 3D), (iv) the ventromedial hypothalamic nucleus (VMH; Fig. 3E), and (v) Amb X (Fig. 3G). The average percent soma area covered by silver grains (taken from 20 neurones for each region in antisense labelled tissue) was corrected for background by subtracting the average obtained from the adjacent sense-exposed section. For the first four regions listed above, the percent area covered by silver grains was arcsin transformed to correct for nonhomogeneity of variance and then analysed by ANOVA (Statview) to compare across sex (between subjects) and among brain areas (within subjects). Post-hoc comparisons between brain regions were done with the Bonferroni/Dunn test. The data collected on the percentage of labelled cells met the assumptions for an ANOVA, so untransformed data were analysed using the same statistical tests. Amb X (Fig. 3G) was not included the statistical analyses because it could not be assessed in all individuals ($n=4$ of each sex). However, because of its importance in controlling dewlap extension, the data obtained for this region are reported in the Results section.

Immunohistochemistry

Animals were euthanized with Sodium Brevital and perfused with PBS followed by 4% paraformaldehyde in PBS. The brain was then removed and treated in one of the following ways: (i) long fixation: postfixed in 4% paraformaldehyde for one hour, embedded in a gelatin block and sunk in

4% paraformaldehyde/20% sucrose overnight; or (ii) short fixation: postfixed in 4% paraformaldehyde for 10 min, embedded in gelatin and sunk in 4% paraformaldehyde/20% sucrose for 3 h. All tissue was sectioned frozen at 25 μ m in the coronal plane and stored in either PBS at 4 $^{\circ}$ C (if reaction occurred within 24 h) or cryoprotectant at -20 $^{\circ}$ C (if tissue was reacted later). Brain sections from intact, breeding males ($n=7$) and females ($n=3$) were prepared using the long fixation protocol, and individuals of both sexes were processed together for immunohistochemistry. Only intact, breeding males ($n=4$; hindbrain sections missing for 1) were subjected to the short fixation procedure. In addition, one male from the nonbreeding season (October; testes were regressed) was implanted for 11 days with a subcutaneous Silastic capsule containing testosterone propionate (0.76 mm inner diameter, 1.65 mm outer diameter, 7 mm long). The brain was subjected to the short fixation protocol, and the results are described separately below.

All washes and incubations were done on a shaker table at room temperature, unless indicated otherwise. Brain sections were rinsed in 0.1 M PBS (pH 7.4) three times (or 10 times if tissue was stored in cryoprotectant) and incubated for 30 min in 0.1 M glycine. Following another series of PBS rinses, the tissue was incubated in 0.5% H_2O_2 for 30 min, rinsed in PBS, blocked for 1 h with 4% normal donkey serum diluted in 0.1 M PBS with 0.2% Triton X-100, and incubated for 48 h at 4 $^{\circ}$ C in the rabbit primary antibody PG-21 (2 μ g antibody/mL of 4% normal donkey serum in 0.1 M PBS with 0.2% Triton X-100). This antibody was raised against the first 21 amino acids of the rat and human androgen receptor (27) and has been successfully used in diverse vertebrate species (see Discussion). Excess primary antibody was removed with a series of PBS rinses, and the brain sections were incubated overnight at 4 $^{\circ}$ C in biotinylated secondary antibody (donkey antirabbit, 1 : 500

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in 0.1 M PBS with 0.2% Triton-X 100; Jackson Laboratories, West Grove, PA, USA). The tissue was rinsed again in PBS and placed in ABC solution (Vector Laboratories, Burlingame, CA, USA, Elite kit) for 2 h. After washes in PBS and Tris buffered saline (pH 7.6), sections were incubated in DAB chromagen (containing 0.002% H₂O₂), rinsed in PBS, mounted on gelatin-coated slides, dehydrated and coverslipped out of xylene. To aid in anatomical localization, alternate sections in some animals were stained with thionin before dehydrating and coverslipping.

Two types of controls were used: (i) the primary antibody was omitted, and the tissue remained in the blocking solution during the time that alternate sections were exposed to PG-21, or (ii) the primary antibody was preadsorbed with 20 times molar excess of the peptide against which it was raised (AR-21) or preincubated with a different region of the AR peptide (AR-462) before its application to the tissue sections. In no primary and AR-21 preadsorbed conditions, no labelling was observed (Fig. 4F). However, preincubation with AR-462 did not interfere with the typical staining pattern.

The distribution of AR-ir was visualized by light microscopy. Within each cell, labelling was characterised as one of the following: (i) nuclear – a smooth reaction product contained within the nucleus of the cell, occasionally with punctate reaction product immediately surrounding and/or overlaying it (Figs 4A,B); (ii) somal – labelling within the cytoplasm and occasionally associated proximal processes, but not in the nucleus (Figs 4C,D); or (iii) in fibers – generally punctate, typical of immunolabelling of peptides (Fig. 4E). Punctate labelling associated with the nucleus probably corresponds to nucleoli, which was previously observed with the same antibody in the fence lizard brain (28). Anatomical localization of labelling was determined using the same maps as for the *in situ* hybridization part of the study (see above).

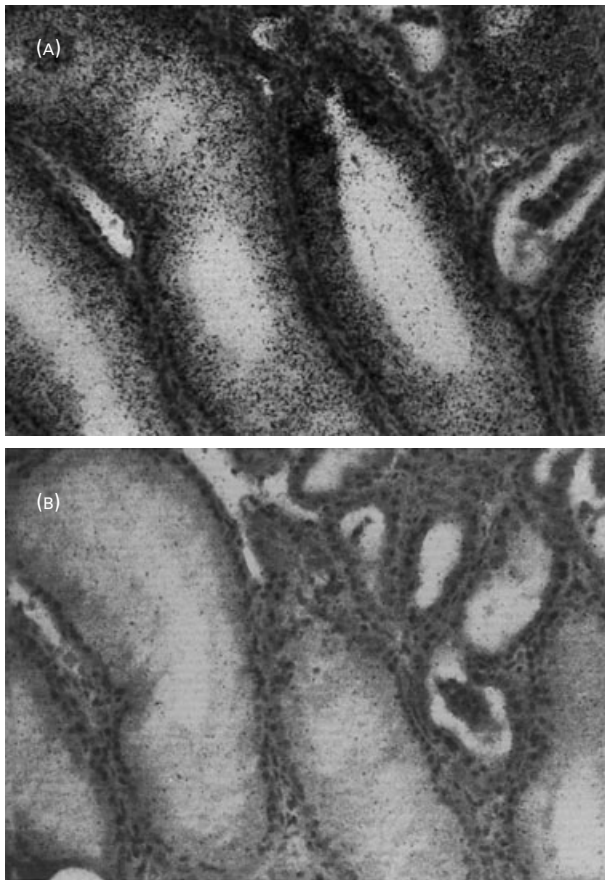


FIG. 2. AR mRNA expression in the renal sex segment of the kidney. (A) Sections exposed to the antisense probe contained extensive, specific labelling in the epithelial cells of the tubules. (B) Sense-exposed sections did not exhibit labelling. Scale bar = 50 μ m.

Results

Cloning and sequencing

A partial nucleotide sequence (697 bp) of the green anole AR was obtained, which included part of the C, all of the D and part of the E domains (Fig. 5; GenBank accession #AF223224). These three domains, respectively, code for the DNA binding, hinge, and hormone binding regions. The predicted amino-acid sequence of the anole AR shares a high degree of similarity with a similar region of AR cloned from humans (94%; (29)), whiptail lizards (92%; (21)), African Clawed frogs (89%; (30)) and rainbow trout (80%; (31)).

In situ hybridization

Cells expressing AR mRNA occurred in discrete regions, from the forebrain through the brainstem, without obvious sex differences in distribution. In general, AR mRNA-containing cells were consistently observed in limbic structures and reticular and motor areas of the brainstem (Fig. 3), including the motor nucleus of cranial nerve V (which is not depicted). Labelling was also seen in the cervical spinal cord (Fig. 3H). Among the brain regions potentially relevant to reproductive behaviours, a significant effect of area was observed ($F = 14.47$, $P < 0.001$), such that labelling was more intense, in the VMH and VMN-amy, than in each of the two other areas (Bonferroni/Dunn test, all $P \leq 0.001$; Fig. 6). The intensity of labelling was also greater in females than males ($F = 5.45$, $P = 0.044$), which was best reflected in the POAp (Fig. 7) and VMN-amy. However, there was no significant interaction between sex and brain region ($F = 0.749$, $P = 0.533$). The percent soma area covered by silver grains in AmbX was similar to that in the MPOA (male mean \pm SE = $3.8 \pm 0.4\%$; female = $4.7 \pm 0.9\%$). With respect to the percentage of labelled cells, there was an overall effect of brain area ($F = 8.77$, $P < 0.001$) such that the VMH and VMN-amy contained a greater percentage of labelled cells than the other two areas (Bonferroni/Dunn test, all $P \leq 0.005$). The main effect of sex ($F = 0.017$, $P = 0.900$) and its interaction with brain region ($F = 1.32$, $P = 0.288$) were not significant (Table 1).

TABLE 1. Percentage of cells (mean \pm SEM) expressing mRNA in areas potentially associated with sexual behaviours in anoles.

Brain area	Male ($n = 5$)	Femal ($n = 6$)
POAp	57.0 \pm 7.8	65.0 \pm 17.9
MPOA	69.0 \pm 16.0	53.3 \pm 24.2
VMN-amy	78.0 \pm 14.4	85.0 \pm 16.7
VMH	89.7 \pm 2.1	87.5 \pm 4.2
Amb X*	44.3 \pm 5.5	43.8 \pm 7.5

*Amb X could only be measured in four individuals of each sex due to histological artifact and was therefore omitted from statistical analyses. POAp, posterior preoptic area; MPOA, medial preoptic area; VMN-amy, ventromedial nucleus of the amygdala; VMH, ventromedial hypothalamic nucleus; AMb X, vagal portion of nucleus ambiguus.

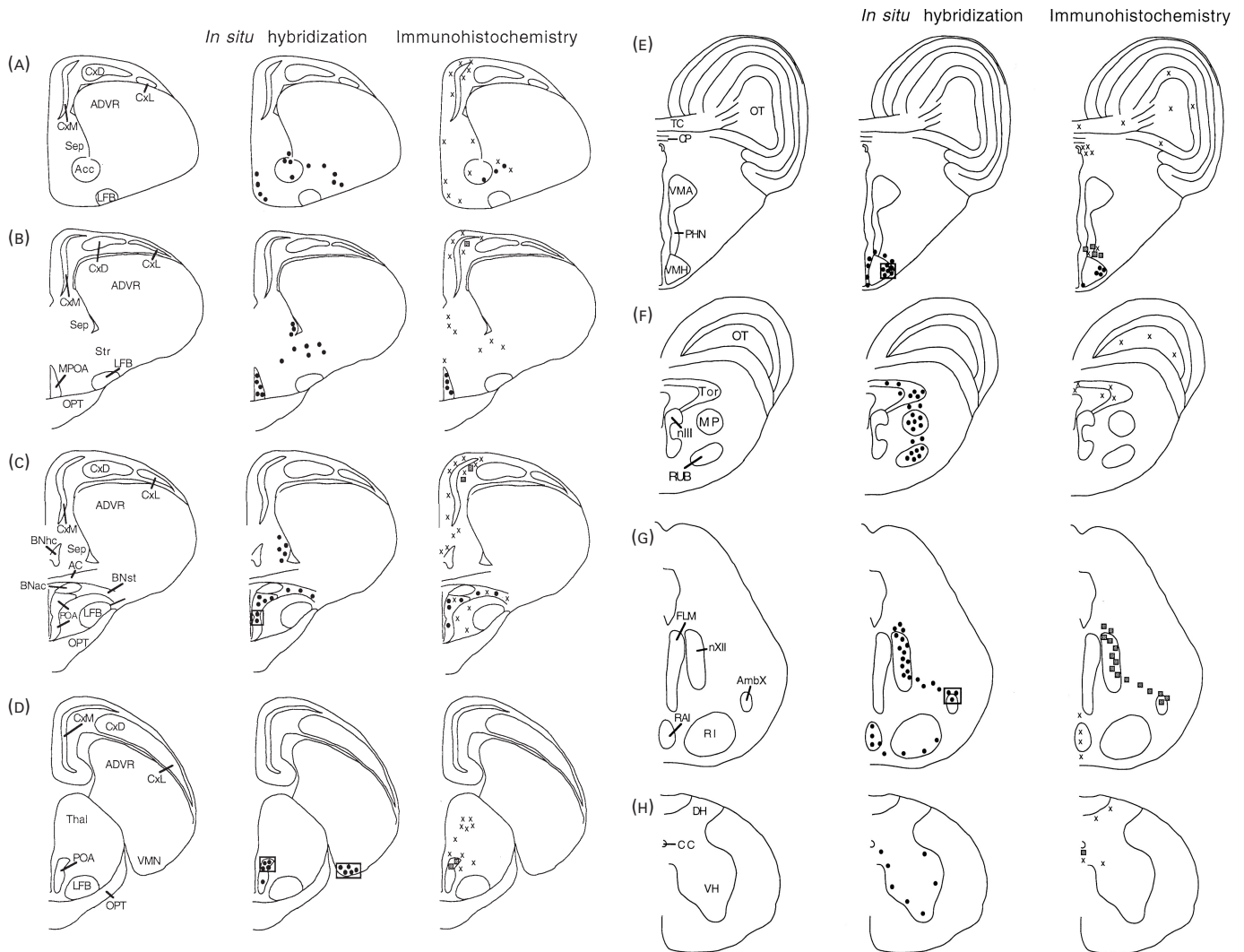


FIG. 3. Camera lucida drawings depicting the distribution of AR mRNA and immunoreactivity in the brain (only half the brain is shown; the distribution was symmetrical). Levels of mRNA expression were quantified in the boxed regions in C, D, E, and G (see Materials and Methods). Solid circles = nuclear labelling; grey squares = soma labelling; x = fibre labelling. Abbreviations: Acc = nucleus accumbens; AC = anterior commissure; ADVR = anterior dorsal ventricular ridge; Amb X = vagal portion of nucleus ambiguus; BNac = bed nucleus of the anterior commissure; BNhc = bed nucleus of the hippocampal commissure; BNst = bed nucleus of the striatum; cc = central canal; CP = posterior commissure; CxD = dorsal cortex; CxL = lateral cortex; CxM = medial cortex; DH = dorsal horn of spinal cord; FLM = medial longitudinal fasciculus; LFB = lateral forebrain bundle; MP = medial pretecal nucleus; MPOA = medial preoptic area; nIII = oculomotor nucleus; nXII = hypoglossal nucleus; OPT = optic tract; OT = optic tectum; PHN = posterior hypothalamic nucleus; POA = preoptic area; RAI = inferior raphe nucleus; RI = reticular nucleus; RUB = red nucleus; Sep = septum; Str = striatum; TC = tectal commissure; Thal = thalamus; Tor = torus semicircularis; VH = ventral horn of spinal cord; VMA = ventromedial area; VMH = ventromedial nucleus of hypothalamus; VMN = ventromedial nucleus of amygdala.

Immunohistochemistry

The distribution of AR-ir closely paralleled the data obtained by *in situ* hybridization (Fig. 3). However, some areas contained AR-ir, but lacked labelling for mRNA. In particular, AR-ir somata were observed consistently in the medial cortex (Figs 3B,C), and lateral to (but not in) nucleus rotundus (not depicted). Fibers were routinely observed in the medial cortex (Figs 3A–C), medial septum (Figs 3A–C), thalamus (Fig. 3D), optic tectum (Figs 3E,F), and in the dorsal funicular nucleus, immediately anterior and posterior to area postrema (not depicted). They also consistently occurred dorsal to the bed nucleus of the hippocampal

commissure (Fig. 3c), around the ventral border of the habenula (Fig. 4E), and as an extensive network surrounding the interpeduncular nucleus and fourth ventricle in the medullary tegmentum (not depicted). Although sections through the rostral spinal cord were present in only a few individuals, a few darkly labelled somata and associated processes were consistently detected along the midline, immediately ventral to the central canal (Fig. 3H). Conversely, some regions lacked AR-ir, but contained labelling by *in situ* hybridization: the VMN-amy, medial pretecal nucleus, nucleus ruber, motor nucleus of cranial nerve V, reticular nucleus, and the motor plate of the cervical spinal cord (Figs 3D,F–H).

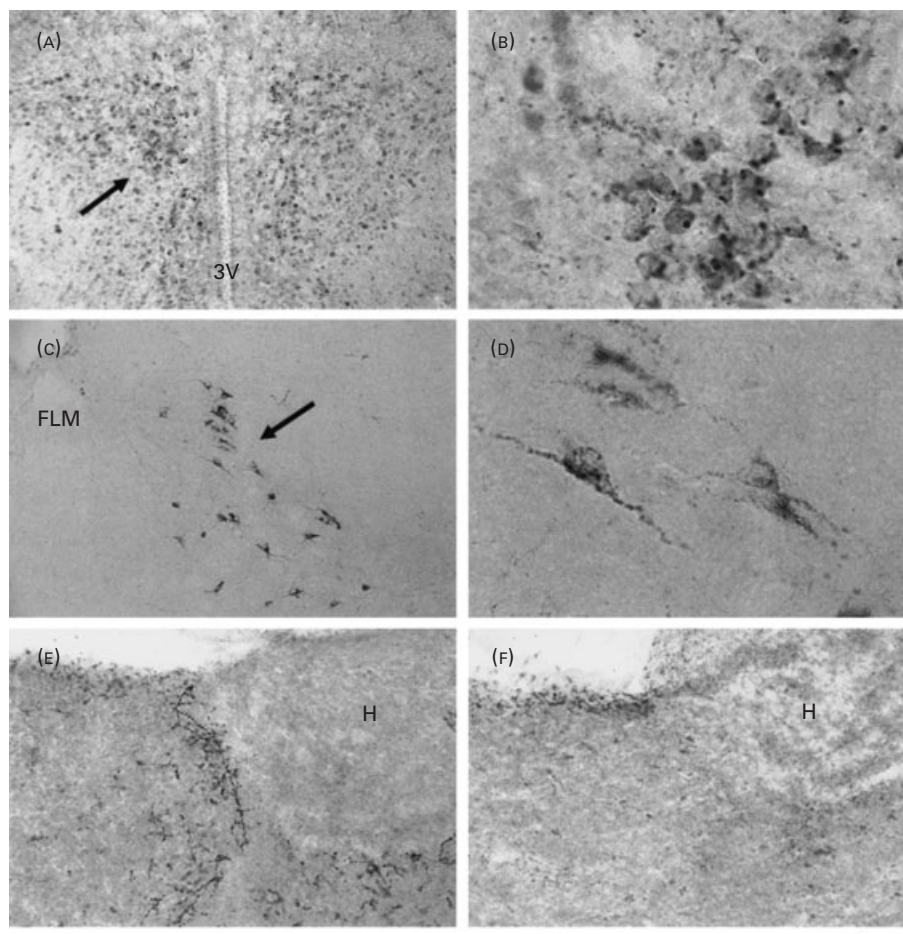


FIG. 4. Intracellular compartmentalization of AR-ir in the green anole brain. Panel A depicts nuclear labelling in the preoptic area, and the arrow indicates the region shown at higher magnification in B. C depicts AR-positive cells in the hypoglossal nucleus. The arrow indicates cells shown in D at higher magnification. Panel E shows AR-positive fibres surrounding the ventral border of the habenula, and the preadsorbed control of the same region is shown in panel F. 3 V = third ventricle; FLM = medial longitudinal fasciculus; H = habenula. Same scale bar = 100 μ m in A, C; 20 μ m in B, D; and 50 μ m in E, F.

The cellular distribution of immunoreactivity appeared to depend on fixation time. That is, brains from the short fixation protocol had labelling primarily in neuronal nuclei, whereas brains from the longer fixation protocol had immunoreactivity localized in soma and fibres. Interestingly, the male treated with testosterone propionate exhibited both nuclear and fibre staining, and, in addition to what was observed in the other brains, contained labelling in areas that corresponded to mRNA expression – nuclear labelling in the lateral septum, and nuclear and fibre labelling ventromedial to nucleus rubar (neither depicted).

Discussion

Both AR-ir and mRNA expression were detected in discrete areas throughout the brain, including structures of the limbic system and brainstem motor nuclei. This distribution of AR-ir and mRNA-labelling in the green anole is consistent with the pattern of androgen binding sites identified in this species by autoradiography (13), with one apparent exception. In the present study, AR-ir and mRNA were consistently observed in the hypoglossal nucleus, but labelling of this nucleus was

not reported in the autoradiography study. The reason for this difference is unclear, but may be due to differences in circulating androgen between individuals in the present and previous studies. Lizards in the autoradiography study were taken from the nonbreeding season and had been castrated long before injection of the labelled steroid, and thus probably had very low levels of circulating androgen. In contrast, all lizards from the present study were intact and, with the exception of one male that had been treated with testosterone propionate, were sacrificed during the breeding season. Because androgen treatment can increase detectable AR in other species (15–18), the higher levels of circulating testosterone in the lizards in the present study may have resulted in greater expression of AR.

Compared to the results with *in situ* hybridization and autoradiography (13), the distribution of AR-ir nuclei and somata was less extensive. The antibody used in the present study (PG-21) was raised against the first 21 amino acids of the rat/human AR, so possible species differences in this region (the full anole cDNA was not cloned) or modifications following translation might have resulted in detection of less AR peptide in the anole. However, the lack of labelling in two

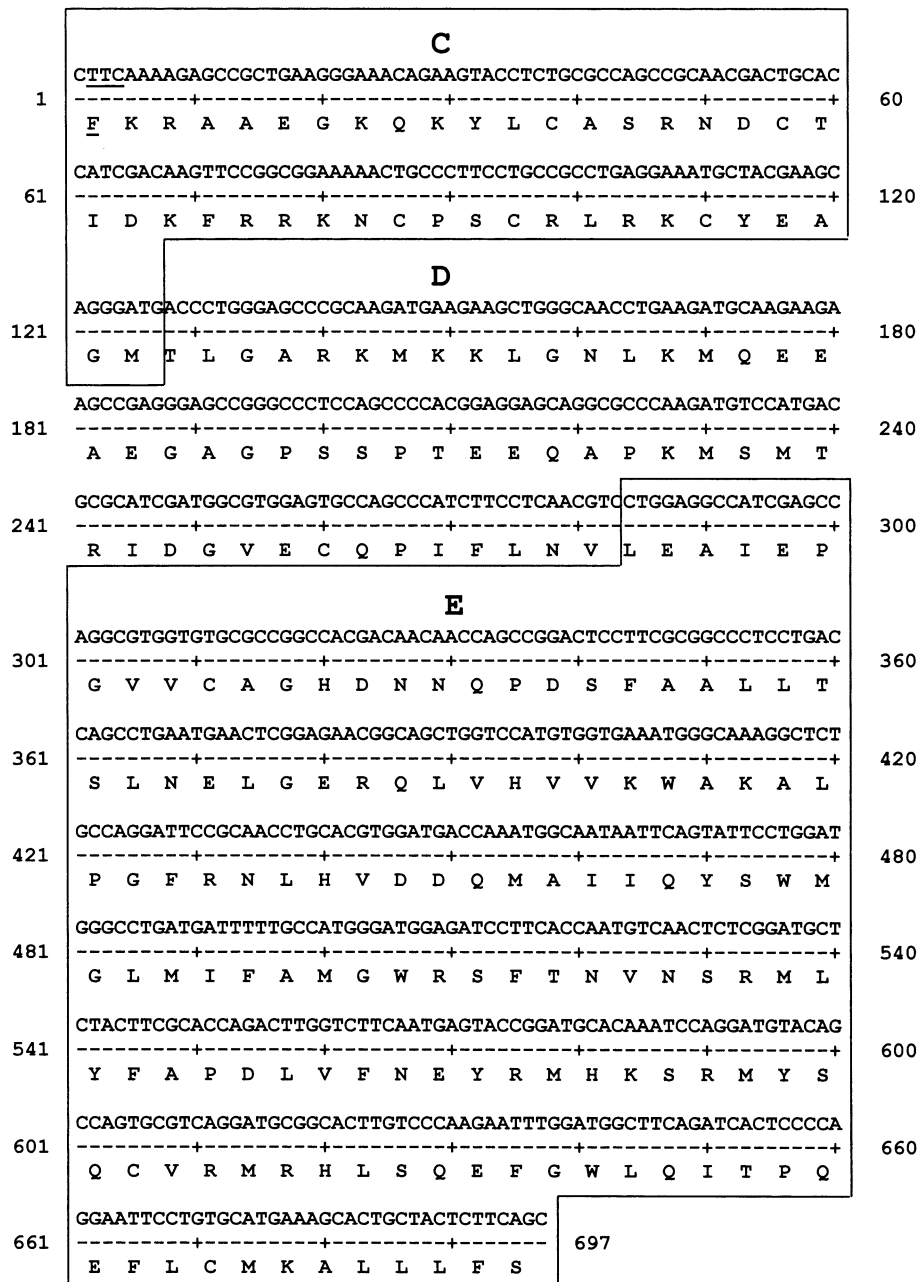


FIG. 5. Partial nucleotide and predicted amino acid sequence of the green anole AR. The DNA binding, hinge, and hormone binding domains are indicated by the letters C, D and E, respectively. The first three nucleotides and corresponding amino acid residue of the protein sequence are underlined.

types of control tissue sections and the fact that the primary antibody, PG-21, has been successfully used in frogs (32), lizards (28), birds (33), rodents (34, 35) and humans (36) provide strong support for the labelling in the present study being specific to the androgen receptor.

Comparison with other species

The expression pattern of AR-ir and mRNA in green anoles is remarkably similar to that detected by various techniques in diverse vertebrate groups, ranging from fish to mammals. In particular, localization of AR to regions of the POA and

hypothalamus, as well as other limbic structures (28, 37–41), is consistent with the highly conserved function of these regions in facilitating reproductive and aggressive behaviours (42–45). The presence of AR in midbrain auditory nuclei (e.g. torus semicircularis) also appears to be evolutionarily conserved, as similar labelling has been detected in the oyster toadfish (46), roughskin newt (38), African clawed frog (47), and garter snake (39). In addition to the anole, AR has been detected in the hypoglossal nucleus of the rat via *in situ* hybridization (41) and immunohistochemistry (48) and in various avian species via autoradiography (49–51), immunohistochemistry (33), and *in situ* hybridization (52). The caudal

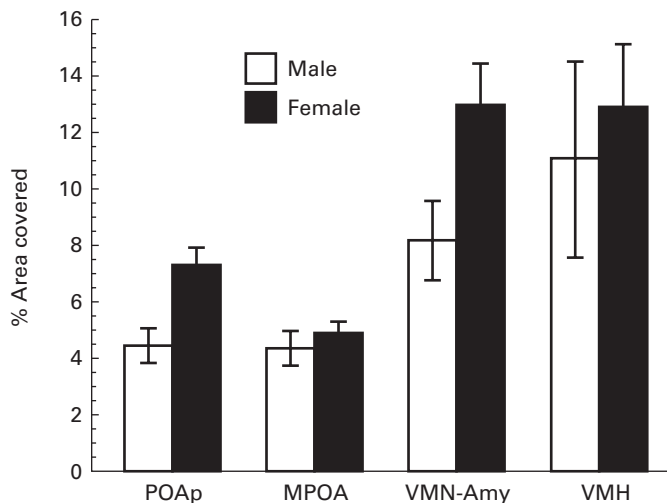


FIG. 6. Mean \pm SE of the percent soma area covered by silver grains in regions likely to mediate sexual behaviours. Labeling was significantly greater in the VMN-amy and VMH than in the other brain areas, and greater overall in females compared to males. POAp, posterior preoptic area, dorsal portion; MPOA, medial preoptic area; VMN-amy, ventromedial nucleus of the amygdala; VMH, ventromedial nucleus of the hypothalamus.

two-thirds of the hypoglossal nucleus innervates the syrinx in birds, which is responsible for song production (53, 54). The presence of the receptor in the hypoglossal nucleus in different species is intriguing because a behavioural role for androgen in this area is obvious only for song birds.

The intracellular location of AR-ir in anoles is consistent with that detected in other vertebrate species; AR-ir in the cytoplasm of cell bodies and processes has been reported in the Syrian hamster (15, 35), and more recently in the fence lizard (28). Similar labelling of oestrogen receptors has been studied in detail in the guinea pig (55, 56). The remarkable similarity in the distribution and appearance of AR-ir-containing fibres in the green anole and western fence lizard (28) suggests that the processing and/or action of AR in lizards might occur in diverse regions of the cell. It is not clear whether the immunoreactivity represents occupied or unoccupied receptors, or even functional protein, however, the consistency with the autoradiography data implies that the neuronal regions do bind androgens.

Potential functional significance

The presence of AR-ir and mRNA in regions of the POA and in the VMN-amy is consistent with the function of these areas in mediating androgen dependent behaviours in male anoles. Lesions in these areas diminish courtship display (8, 9, 57), and implants of testosterone or dihydrotestosterone directly into the AH-POA restore the behaviour in males (4, 7). The enhanced expression of AR in females compared to males was unexpected, as sex differences in other vertebrate species; the distribution or level of AR expression is generally more extensive in males than females (16, 28, 35, 48, 58). However, the effect is not uniform across regions, and it is possible that

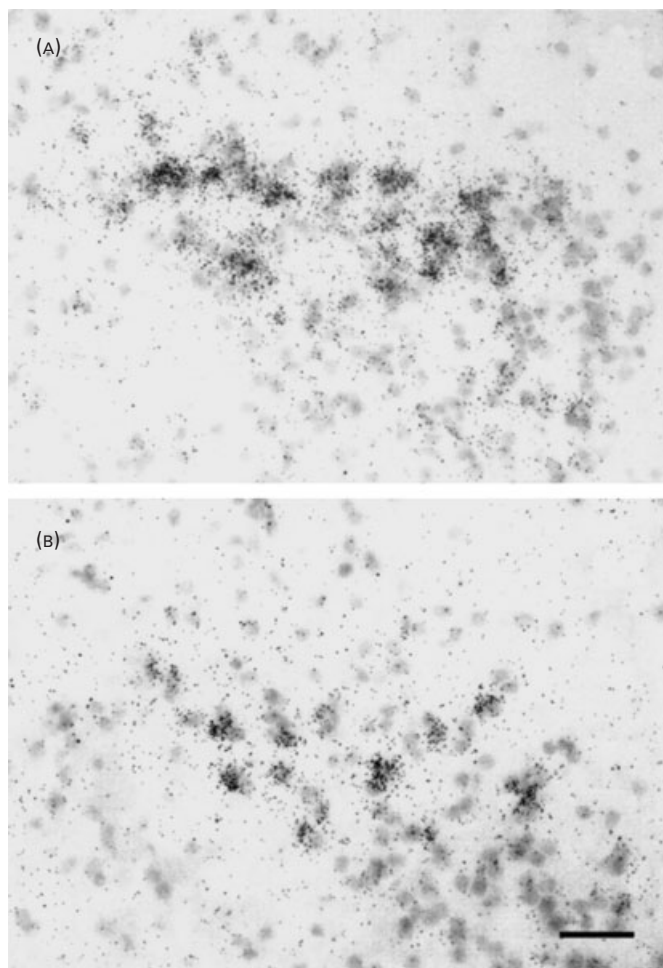


FIG. 7. AR mRNA expression in the posterior preoptic area (POAp) from a female (A) and male (B). The photos correspond to the medial boxed region in Fig. 3d. Scale bar = 30 μ m.

the mRNA is not as efficiently translated in females as in males, so the AR protein levels may not differ. In any case, because the functions are currently unknown for the VMN-amy in females and the POAp in both sexes, the behavioural significance of AR in these regions is unclear.

The similar distribution and equivalent percentage of cells expressing AR in the two sexes suggest that androgens might play a role in female, as well as male, sexual behaviour. Although estradiol and progesterone are the primary circulating gonadal steroids in female anoles (59), testosterone is higher in breeding females than nonbreeding females (60), and systemic treatment with testosterone facilitates receptivity in gonadectomized females (3, 6). While this effect is at least due in part to aromatization (6), the expression of AR mRNA in the limbic system of females may permit the low circulating levels of testosterone to facilitate their behaviours, either directly, or perhaps by enhancing the effects of estradiol and progesterone on receptivity (61). AR in the VMH may be particularly important for receptivity, as lesioning this area in other lizard species inhibits the behaviour (62).

While some differences in receptor distribution are expected among brain regions, we were surprised that AR-ir or mRNA was detected only at relatively low levels in the main motor areas responsible for extension of the dewlap (11). Although a very small nucleus, relatively few cells were lightly labelled for AR-ir and mRNA in Amb X, and none were detected in Amb IX/VImv. These caudal brainstem regions are very similar to the location of the motoneurons responsible for androgen-sensitive behaviours in other species, such as masculine frog vocalizations (47) and head-bobbing associated with crowing in quail (52). Given the greater expression of mRNA in the forebrain regions of anoles, perhaps androgens exert their primary influence on higher centres in the brain (e.g. POA, VMN-amy) to activate, or perhaps increase motivation for, masculine behavioural displays.

The function of AR in the other brainstem areas is unclear. Some areas might mediate aspects of display behaviour that have yet to be tested in the anole. Another possibility is that T plays a more general role in supporting motoneurone structure. For example, in the spinal nucleus of the bulbocavernosus of adult rats, peripheral treatment with T increases soma size (63, 64) and the number of synaptic inputs (65). Androgens also facilitate peripheral nerve regeneration of the facial nerve following nerve crush (66).

Conclusions

The present results are consistent with the neuroanatomical distribution of AR previously detected in anoles by autoradiography. Together, these studies demonstrate the presence of AR in areas likely to directly mediate androgen-dependent behaviours, such as the POA, VMH and VMN-amy. In addition, AR occurred in many brainstem areas for which the behavioural function has yet to be confirmed in anoles. Androgen might act on some of the regions to facilitate specific aspects of reproduction, or it might play a more general role in maintaining motoneurone structure or function. The presence of AR-ir in fibers, in addition to nuclei and somata, suggests that the processing and/or action of AR might occur in diverse regions of the cell in anoles, as in mammalian species. The similarity in the distribution of AR among numerous vertebrate species is interesting, as the reproductive importance of particular structures (e.g. some brainstem motor nuclei) varies across species and thus regional utilization of androgen may be expected to differ. A conserved distribution of AR in many areas of the brain may have allowed animals to capitalize on different sensory and motor modalities for communication.

The lack of sex differences in the distribution of AR-ir and mRNA labelling, as well as relatively few (and female-biased) differences in expression levels of mRNA, are consistent with the idea that male typical behaviours in anoles primarily depend on high levels of circulating testosterone, rather than enhanced androgen sensitivity. These results parallel those seen with steroid metabolizing enzymes. That is, while conversion of testosterone to dihydrotestosterone is important for maximal display of courtship in male anoles (5), 5 α -reductase activity in the brain is generally equivalent between males and females in the breeding season and between breeding and nonbreeding males (67). Thus, anoles might represent a basic

system, or perhaps a primitive condition, in which circulating levels of T, rather than differences in androgen sensitivity or metabolism, dictate behavioural dimorphisms.

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