Androgen receptor-immunoreactivity in the forebrain of the Eastern Fence lizard (Sceloporus undulatus)

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Abstract

Androgen receptor (AR) distribution in the lizard forebrain and optic tectum was examined using PG21 immunohistochemistry. In the male Eastern Fence lizard, AR-immunoreactive (-ir) nuclei were observed in the medial preoptic area, ventromedial and arcuate hypothalamic nuclei, periventricular hypothalamus, premammillary nucleus, bed nucleus of the stria terminalis, and ventral posterior amygdala. Punctate immunostaining of neuronal processes (axons and/or dendrites) was concentrated in the cortex, hypothalamus, and optic tectum. AR-ir nuclei in the female brain were confined to the ventral posterior amygdala and ventromedial hypothalamic nucleus. The AR distribution in the lizard brain is similar to that reported for other vertebrate classes. Sex differences in AR-immunoreactivity may contribute to sex-specific behaviors in the Eastern Fence lizard. © 2000 Elsevier Science B.V. All rights reserved.

1. Introduction

Male reproductive behavior is activated by circulating sex steroids, particularly the androgens testosterone and 5α-dihydrotestosterone (DHT). Androgens elicit sex-specific behaviors by acting directly on androgen receptors in the brain or through indirect activation of brain estrogen receptors via androgen aromatization [32]. In whiptail and anole lizards, androgens are more effective than estrogenic metabolites in eliciting male sexual behavior [14,38,42].

The distribution of androgen receptor (AR) protein and mRNA in the brain shows a similar pattern among the different vertebrate classes, including bird [2,36], fish [19], reptile [46], and mammal [21,35]. In each of these classes, AR-positive cells are concentrated in the hypothalamus and amygdala, specifically in brain nuclei that have been implicated in male reproductive and aggressive behavior. Comparative studies have proven useful in delineating species-specific AR-ir cell clusters, such as the song control nuclei of songbirds [1,2], and have increased our understanding of the neural basis of reproductive behaviors [20].

To date, there has been no immunocytochemical study of AR in the reptilian brain. An early study by Morrell et al. [26] employed 3H-testosterone autoradiography to identify androgen-concentrating cells in the lizard brain. Some of the androgen-concentrating cells which they identified may actually have been estrogen-concentrating cells, as circulating testosterone is aromatized to estrogen in certain areas of the brain. Recently, Young et al. [46] analyzed the distribution of AR mRNA in the brain of the whiptail lizard using in situ hybridization. Although highly sensitive, this method cannot distinguish between cytoplasmic and nuclear staining, or identify AR-positive dendrites and axons. In the present study, we map the distribution of AR in the forebrain of the Eastern Fence lizard (Sceloporus undulatus) using immunocytochemistry.

The AR antibody employed in the present experiments (PG21) is directed against amino acids 1–21 of the rat AR [30]. The lizard AR shows a high degree of sequence
homology with ARs in other species [47]. The DNA-binding and C-terminal ligand-binding domains of AR are the most highly conserved [37]. However, positions 1–35 and 230–268 in the AR are far less variant than the rest of the N-terminal domain [37]. A low degree of variance in amino acids 1–21 of the AR may explain the ability of the PG21 antibody to label ARs in diverse tissue types in a variety of vertebrates, including mammal [11,13,18,21,44,45,49], fish [15], bird [2,36], and amphibian [16].

2. Methods

Male (n=6) and female (n=5) lizards (Sceloporus undulatus) were purchased from Charles D. Sullivan Co., Inc. (Nashville, TN) during the spring/summer breeding season (May through September). Nine of the lizards (5 males, 4 females) were classified as S. undulatus consobinus (Southern Prairie subspecies); the other two individuals (experiments L8 and L18) were identified as S. undulatus garmani (Northern Prairie subspecies). Animals were maintained in captivity for 2–8 weeks in glass aquaria (2 to 3 animals per aquarium) with peat moss as substrate, and sticks and stones for climbing. Photoperiod was maintained at 12 h light: 12 h dark. Room temperature was 21–23°C, with additional heat (up to 38°C) provided by a heat lamp situated at one end of the terrarium. Water was available at all times. Animals were fed crickets 2 to 3 times per week, supplemented with reptile vitamins.

At the time of sacrifice, animals were given an overdose of sodium pentobarbital followed by decapitation. The dorsal skullcase was removed, and the brain was immersion-fixed in situ in 4% paraformaldehyde 0.1 M phosphate buffer, pH 7.4, for 5–12 days. Following fixation, the brains were removed from the skull and embedded in 10% gelatin. The gelatin-brain blocks were fixed in 4% paraformaldehyde for an additional 4–6 days. Next, the brains were cut into 20 μm sections on a Vibratome. One series of sections (one-of-three) was processed for AR-immunocytochemistry, and a second series was mounted on gel-coated slides and Nissl-stained with thionin.

The immunocytochemical method was as follows. Sections were treated with 0.3% hydrogen peroxide in 0.01 M phosphate-buffered saline (PBS), pH 7.4, for 10 min, rinsed in PBS, treated with 0.5% sodium borohydride in PBS for 5–10 min, and preincubated for 1 h in a PBS solution containing 2% normal donkey serum (NDS) and 0.3% Triton X-100 (TX). Sections were then incubated with the AR antibody (PG21), diluted 1:1,000–1:5,000 (0.2–1.0 μg/ml, final concentration) in PBS-NDS-TX, for 3–6 days at 4°C. Next, the sections were rinsed in PBS, incubated with a secondary antibody (biotin–SP-conjugated donkey anti-rabbit IgG; Jackson ImmunoResearch; diluted 1:200 in PBS-NDS-TX) for 1–2 h at room temperature, rinsed again, and reacted with ABC reagent (Elite Standard ABC kit; Vector) for 1–2 h. After several rinses in PBS, the sections were reacted in a solution of 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma)-0.025% nickel chloride-0.01% H2O2 in 0.1 M Tris buffer, pH 7.4, for 10 min. The sections were mounted on gel-coated slides, dehydrated through alcohols and xylene, and coverslipped with Permount (Fisher).

The specificity of the PG21 antibody was tested by, (1) preabsorption of the primary antibody with its respective peptide (AR21; amino acids 1–21 of the rat AR), (2) preabsorption of the primary antibody with a peptide derived from a different portion of the rat AR (AR462; amino acids 462–478), and (3) omission of the primary antibody. In the two preabsorption controls, sections were incubated in 1 ml of diluted antibody (1:5000) preincubated with 370 ng of peptide (AR21 or AR462). No immunocytochemical staining was observed in controls where, (1) the primary antibody was omitted, or (2) the primary antibody was preincubated with its respective peptide (AR21). Staining was intact in control experiments involving preabsorption with the distant, unrelated peptide (AR462).

The distribution of AR-immunoreactivity in representative sections was plotted on a microscope (BH-2, Olympus) with a digital stage readout head attached to a computer with Neuroulucida software (MicroBrightField, Inc.). A camera lucida was used to add cytoarchitectural details obtained from adjacent Nissl-stained sections.

3. Results

AR-immunoreactivity was observed in select brain areas in both male and female S. undulatus. The distribution of AR-immunoreactivity in the male Eastern Fence Lizard brain was examined in six experiments. One male lizard (experiment L2) was obtained and sacrificed in July, 1998; two males (experiments L8 and L24) were obtained in September, 1998 and sacrificed in November, 1998; and three males (experiments L21, L22 and L25) were obtained and sacrificed in June, 1999. The pattern of AR-labeled cells and the intensity of the immunolabeling varied by experiment. The following description specifically refers to representative experiment L8, which exhibited the most robust and widespread labeling.

At rostral levels in experiment L8, AR-immunoreactivity was concentrated in the medial and dorsal cortices (Figs. 1A–D; 2A–B). AR fibers in the medial cortex were organized in a bilaminar pattern with both layers juxtaposed to the principal neuron layer (Fig. 2A). In the dorsal cortex, AR fibers displayed numerous varicosities (Fig. 2). At more caudal levels, AR fibers were also found in the lateral cortex (Fig. 1B, C). Cytoplasmic staining was observed in a small number of neurons scattered in the dorsal cortex. Ventral to the medial cortex, there was a
Fig. 1. A series of camera lucida drawings illustrating the distribution of androgen receptor-immunoreactive nuclei (triangles) and fibers (small dots) in the brain of a male Eastern Fence lizard (L8, rostral to caudal, A–E). AHA, anterior hypothalamic area; Arc, arcuate nucleus; AT, area triangularis; BST, bed nucleus of the stria terminalis; DB, nucleus of the diagonal band; DCx, dorsal cortex; DL, dorsolateral thalamic nucleus; DLH, dorsolateral hypothalamic nucleus; DM, dorsomedial thalamic nucleus; DVR, dorsal ventricular ridge; ExA, external nucleus of the amygdala; fb, forebrain bundles; fr, fasciculus retroflexus; Hb, habenula; LA, lateral nucleus of the amygdala; LCx, lateral cortex; LG, lateral geniculate nucleus; LH, lateral hypothalamic area; MCx, medial cortex; MPA, medial preoptic area; MPT, medial preptetal nucleus; MS, medial septal nucleus; NS, nucleus sphericus; oc, optic chiasm; opt, optic tract; OTe, optic tectum; PG, pretectal geniculate nucleus; PrM, premammillary nucleus; Pv, periventricular hypothalamus; Rot, nucleus rotundus; S, septum; StA, striatoamygdalar area; TV, tectal ventricle; VMH, ventromedial hypothalamic nucleus; VPA, ventral posterior amygdala.
Fig. 2. Photomicrographs of androgen receptor-ir fibers in the lizard brain (experiments L2 and L8). (A) AR-ir fibers in the medial cortex were concentrated in two layers, one superficial and the other deep to the principal neuron layer (marked with an asterisk). (B) In the dorsal cortex, some AR-ir fibers were oriented perpendicular to the brain surface (principal neuron layer indicated by asterisk) and many fibers were concentrated along the dorsal brain surface. (C) AR-ir labeling in the preoptic area was diffuse and punctate (two AR-ir fibers are indicated by arrows). AHA, anterior hypothalamic area; DCx, dorsal cortex; DVR, dorsal ventricular ridge; MCx, medial cortex; opt, optic tract; 3V, third ventricle. Scale bar=100 μm.
and periventricular hypothalamus were more numerous in and pattern of AR expression (cf., Fig. 1 with Figs. 3 and 4 number of AR-ir nuclei varied by experiment, although likely corresponds to the ventral posterior amygdalar (Figs. 1E; 3D). lary nucleus, and the periventricular hypothalamus. The fibers were concentrated in the deep, ventricular layers thalamic nucleus, the medial preoptic area, the premammil-
nucleus rotundus (Fig. 1E). In the optic tectum, labeled external amygdalar nucleus, the ventromedial hypo-
nucleus [28] and dorsolateral to the caudal portion of the fasciculus retroflexus, ventral to the medial pretectal whiptail lizards, a band of AR-ir labeling was observed in the is similar to the AR mRNA distribution reported for the area, and the ventral posterior amygdala. This distribution did not extend into the gray matter of the thalamus (Fig. 1B); it extended caudally as a thin band of cells ventrolateral to the nucleus sphericus (Fig. 1C).

In the hypothalamus, some lightly stained AR-ir nuclei were observed in the medial preoptic area, particularly in the caudal dorsal portion (Fig. 1B). Scattered AR-ir fibers, punctate in appearance, were present throughout the preop-tic and anterior hypothalamic areas (Fig. 2C). Immediately caudal to the optic chiasm, we observed a concentration of AR-ir fibers in a dorsal section of the periventricular hypothalamus (Fig. 1D). In Nissl-stained sections, this hypothalamic area showed a distinct cytoarchitecture with many small, dark-staining neurons as well as larger, medium-staining neurons. Adjacent areas in the periven-
tricular hypothalamus contained only large, medium-staining neurons. A cluster of lightly labeled AR-ir nuclei was observed the ventromedial nucleus (Fig. 1D). Many darkly stained, AR-ir nuclei with prominent, stained nucleoli were present in the premammillary and arcuate nuclei (Figs. 1E; 3B, C). Scattered AR-ir fibers were found throughout the hypothalamus.

AR-immunoreactivity was observed in a few other areas of the diencephalon. AR-ir fibers were concentrated in a cell-dense area located medial to the lateral geniculate nucleus and dorsal to the forebrain bundles (Fig. 1C); this region corresponded to the area triangularis [9]. Axonal labeling was present throughout the lateral forebrain bundle (Fig. 1B–E). Many AR-ir fibers coursed dorsally through the white matter adjacent to the third ventricle, but did not extend into the gray matter of the thalamus (Fig. 1E). A band of AR-ir labeling was observed in the fasciculus retroflexus, ventral to the medial pretectal nucleus [28] and dorsolateral to the caudal portion of the nucleus rotundus (Fig. 1E). In the optic tectum, labeled fibers were concentrated in the deep, ventricular layers 1–5 (Figs. 1E; 3D).

In the six experiments involving male lizards, the number of AR-ir nuclei varied by experiment, although this was not quantified. AR-ir nuclei in the arcuate nucleus and periventricular hypothalamus were more numerous in experiment L8 (November) than in representative experiments L2 (July) and L21 (June). Conversely, more labeled cells were observed in the bed nucleus of the stria terminalis and striatoamygdalar area in experiment L2 than in L8 or L21. The intensity of the immunolabeling (both AR-ir nuclei and fibers) was greatest in L8, moderate in L2, and least in L21.

AR-immunoreactivity in the female Eastern Fence lizard brain was examined in five experiments. Two female lizards (experiments L17 and L18) were obtained in September, 1998 and sacrificed in November, 1998; and three females (experiments L19, L20 and L23) were obtained and sacrificed in May, 1999. The distribution of AR-immunoreactivity was identical for each case.

In representative experiment L23, AR-ir nuclei were confined to the ventral posterior amygdala (Fig. 4A) and the ventromedial hypothalamic nucleus. Labeled nuclei in these areas were more lightly stained and fewer in number than in the male brain. No nuclear labeling was observed in the medial preoptic area, the arcuate nucleus, the bed nucleus of the stria terminalis, or the striatoamygdalar area in any experiment involving female lizards (Fig. 4B).

The pattern of AR-ir fiber labeling in representative experiment L23 (female) closely resembled that in L8 (male). In both male and female, AR-ir fibers were concentrated in the medial cortex, dorsal cortex, medial septum, area triangularis, dorsal portion of the periven-tricular hypothalamus, habenular nuclei, optic tectum, lateral forebrain bundle, and the white matter surrounding the third ventricle.

4. Discussion

Our results show that AR-immunoreactivity is concen-trated in limbic areas of the lizard forebrain, particularly the medial cortex, amygdala, and hypothalamus, and that this immunoreactivity is sexually dimorphic, with a wider distribution of AR-ir nuclei present in the male brain as compared to the female.

In the male fence lizard, we observed AR-ir cell nuclei in the medial preoptic area, the ventromedial hypothalamic nucleus, the arcuate nucleus, the caudal ventral part of the periventricular hypothalamus, the premammillary nucleus, the bed nucleus of the stria terminalis, the striatoamygdalar area, and the ventral posterior amygdala. This distribution is similar to the AR mRNA distribution reported for the whiptail lizards, *Cnemidophorus uniparens* and *C. inornatus* [46]. In the whiptail lizard, AR is expressed in the external amygdalar nucleus, the ventromedial hypothalamic nucleus, the medial preoptic area, the premammillary nucleus, and the periventricular hypothalamus. The external amygdalar nucleus in the whiptail lizard most likely corresponds to the ventral posterior amygdalar nucleus in the fence lizard, based on its ventral location and pattern of AR expression (cf., Fig. 1 with Figs. 3 and 4.
Young et al. [46] observed several additional AR-positive cell clusters in the whiptail lizard that we did not detect in the Eastern Fence lizard, such as the dorsal lateral thalamic nucleus and the anterior portion of the dorsal ventricular ridge. The in situ hybridization method employed by Young et al. [46] is more sensitive than immunocytochemistry, which could account for the additional AR cell populations detected in their study. Alternatively, there may be species differences in AR expression between whiptail and fence lizards.

The distribution of AR-immunoreactivity in the lizard brain is similar to that observed in the brains of other vertebrates. In the mammalian brain, AR-ir nuclei are present in the bed nucleus of the stria terminalis; the
Fig. 4. Photomicrographs of AR-ir nuclei and fibers in the female lizard brain (experiment L23). (A) In the ventral posterior amygdala, most of the AR-ir nuclei were lightly stained. (B) A few AR-ir fibers, but no AR-ir nuclei, were observed in the arcuate nucleus and periventricular hypothalamus. Arc, arcuate nucleus; NS, nucleus sphericus; Pv, periventricular hypothalamus; VPA, ventral posterior nucleus of the amygdala; 3V, third ventricle. Scale bar=100 μm.

medial preoptic area; the arcuate, ventromedial and paraventricular hypothalamic nuclei; the ventral premammillary nucleus; the medial and cortical amygdalar nuclei; the CA1 hippocampus; and the cerebral cortex [10,12,21,34,43,45,49]. A similar pattern is observed in the bird brain, with AR-ir nuclei concentrated in the medial preoptic, paraventricular and ventromedial hypothalamic nuclei; the infundibular hypothalamus (corresponding to the mammalian arcuate nucleus); the nucleus taeniae (avian homologue of the mammalian amygdala); the nucleus intercollicularis; and several telencephalic nuclei involved in birdsong [1,2,36]. In the fish brain, AR-ir nuclei are located predominantly in the dorsal and lateral parts of the ventral telencephalon (possibly corresponding to the mammalian lateral septum and amygdala, respectively [8]), the preoptic, periventricular and tuberal hypothalamus, the dorsal telencephalon, and the optic tectum [19]. Across the different vertebrate classes, AR-ir neurons are found consistently in certain brain areas, such as the amygdala, preoptic area and arcuate hypothalamus; these areas are necessary for reproductive behavior and/or hormone release [24,27,32,41]. AR-immunoreactivity in taxon-specific areas, such as the magnocellular nucleus of the anterior neostriatum [1], the supraoptic nucleus [13], the optic tectum [19] and the nucleus of the lateral olfactory tract [21], may be involved in more specialized aspects of reproductive behavior (e.g., song production, intruder-chasing) or in other adaptive behaviors (e.g., water homeostasis).

We observed dark-staining of the nucleolus in many AR-ir nuclei located in the amygdala and hypothalamus, suggesting that androgens may activate nucleolar processes (e.g., transcription of ribosomal genes) in the lizard brain. Several other studies have noted steroid receptor immunostaining of nucleoli [6,17,23]. For example, Kessels et al. [23] observed nucleolar staining in breast carcinoma cells (MCF7) immunostained for the estradiol receptor. In osteoblasts and osteocytes, an antibody to the steroid 1,25-dihydroxyvitamin D3 receptor labels primarily the nuclear chromatin but also the nucleolus [6]. AR-ir nucleoli have not previously been described, possibly due to masking of nucleolar immunoreactivity by intense nuclear AR-immunoreactivity. In support of this possibility, we could not distinguish AR-ir nucleoli in darkly stained nuclei. Most of the AR-ir cell nuclei in our experiments were lightly stained which we attributed to the relatively
long fixation times (9–18 days) involved in post mortem immersion fixation and gelatin blocking.

AR cytoplasmic labeling was observed with the PG21 antibody in the Eastern Fence lizard brain. The punctate staining pattern of the AR-ir fibers was consistent with a synaptic localization on either axon terminals and/or dendrites. Gial processes may also possess steroid receptors [22], but the length and diameter of the AR-ir fibers in the lizard brain makes a glial origin unlikely. We believe this cytoplasmic immunostaining to be specific for AR for the following reasons. (1) In control experiments, AR-immunoreactivity in both cell nuclei and neuronal processes was completely abolished when the PG21 antibody was preabsorbed with its immunogenic peptide (AR21) or when the primary antibody was omitted. (2) Cytoplasmic staining of steroid receptors is well documented and has been reported for the estrogen [4,5,29], androgen [18,44], progesterin [3,31], and glucocorticoid [33] receptors. In the guinea-pig hypothalamus of ovariectomized females, estrogen receptor-immunoreactivity is present in dendrites and axon terminals, as well as in the cell soma [5]. A punctate staining pattern, similar to that observed in the present study, has been demonstrated for membrane glucocorticoid and estrogen receptors [29,33,40]. Future studies will examine the ultrastructure and possible neuronal origin of the AR-ir fibers in the fence lizard brain.

A sex difference in AR-immunoreactivity was detected in the hypothalamus and amygdala. AR-ir nuclei were abundant in the hypothalamus of the male fence lizard but were restricted to the ventromedial nucleus in the female. AR-immunostaining in the amygdala of the female lizard was limited to the most ventral portion of the ventral posterior nucleus; staining in the male was more extensive and spanned the ventral posterior nucleus, the striatoamygdalar area and the bed nucleus of the stria terminalis. Few studies have examined the brains of both sexes for possible sex differences in AR protein or mRNA expression. Six-day-old male rats show robust AR immunostaining of the ventral premammillary nucleus; this nucleus is unstained in female rats of the same age [45]. In the fetal mouse (E16), an intense AR mRNA signal is present in the male embryo brain with only weak AR signal detected in the female brain [48]. These and the present results suggest that a sex comparison of AR-immunoreactivity in the brain of an adult bird or mammal would be worthwhile.

Sex differences in the brain are generally correlated with sexually dimorphic behaviors [20]. Male and female S. undulatus show sex differences in size, skin coloring, and aggressive behavior [39]. Males have blue belly patches which they display during frequent aggressive encounters. Females are physically larger than males, lack blue coloration on their belly, and display little or no aggression. The marked sexual dimorphism in brain AR-immunoreactivity in this species may underlie sex differences in its behavior.

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References


