Androgen Receptor mRNA Expression in *Xenopus laevis* CNS: Sexual Dimorphism and Regulation in Laryngeal Motor Nucleus

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**SUMMARY**

Using Northern analysis, *in situ* hybridization, and nuclease protection assays, the expression and regulation of androgen receptor messenger RNA (AR mRNA) was examined in the CNS of juvenile *Xenopus laevis*. Only one of the AR mRNA isoforms expressed in *X. laevis* is transcribed in the CNS as shown by Northern blot analysis. Nuclease protection assays demonstrate that the expression of AR mRNA is higher in the brain stem than in the telencephalon and diencephalon. Although expression of AR mRNA is widespread throughout the CNS, cells of cranial nerve nucleus IX–X (N. IX–X) and spinal cord display the highest *in situ* hybridization signals in their cytoplasm. Double labeling using horseradish peroxidase and digoxigenin labeled AR probes reveals that laryngeal and anterior spinal cord motor neurons express AR mRNA. More cells express AR mRNA in N. IX–X of males than of females. The number of AR expressing cells in N. IX–X decreases following gonadectomy in both sexes, and dihydrotestosterone (DHT) treatment for 1 month reverses this effect. Increased expression of AR mRNA in the brain of DHT treated animals is also apparent in nuclease protection assays. Sex differences in number of AR expressing cells and hormone regulation of AR mRNA expression in motor nuclei may influence neuromuscular systems devoted to sexually differentiated behaviors. © 1996 John Wiley & Sons, Inc.

**Keywords:** androgen receptor, mRNA expression, motor neurons, sexual dimorphism.

**INTRODUCTION**

Steroid hormones regulate neuroendocrine functions and behaviors related to reproduction. Among the most dramatic effects of steroids are alterations in developmental programs leading to sex differences in the CNS. In many cases, hormone sensitive neurons have been implicated in the control of sex-specific reproductive behaviors. For example, nuclei of the preoptic area and hypothalamus of mammals have been shown to be sexually dimorphic and are involved in reproductive conduct (Tobet et al., 1986; Paredes and Baum, 1995).

In canaries, sexually dimorphic regions of the telencephalon undergo seasonal modifications in size related to variations in bird song (Nottebohm, 1980). In the frog *Xenopus laevis*, the IX–X nerve differs between sexes in the number of axons that innervate the larynx or vocal organ (Kelley and Dennison, 1990). The larynx itself varies in muscle fiber type and size due to an androgen-driven program of masculinization; sex differences in this neuromuscular system result in sex-specific patterns of vocalization (Kelley and Pfaff, 1976; Hanneken and Kelley, 1986; Watson and Kelley, 1992; Watson et al., 1993). We examined the possibility that nuclei of the brain involved in differentiated behaviors are distinct in males and females in regard to their sensitivity to hormones. Thus, we analyzed in the cranial nerve nucleus (N.) IX–X whether the expression of androgen receptor messenger RNA (AR mRNA) is sexually dimorphic.

Androgens interact with an intracellular recep-
tor that induces gene transcription (Berger and Watson, 1989). In the rat CNS, AR mRNA and protein are widely expressed in regions including the telencephalon, neurons within the cranial nerve nuclei, and motor neurons of the spinal cord (Simerly et al., 1990; Osada et al., 1993; Al Shamma and Arnold, 1995). In X. laevis, two isoforms of AR are expressed in the larynx (Fischer et al., 1993, 1995). In the CNS, diencephalic, mesencephalic, and medullary nuclei, including cells within the N. IX–X and anterior spinal cord, accumulate testosterone and dihydrotestosterone (DHT; Kelley et al., 1975; Kelley, 1980, 1981; Erulkar et al., 1981; Gorlick and Kelley, 1986). An unresolved issue within this system is the identification of cell types that express AR. Anterior spinal cord and N. IX–X include both motor neurons and interneurons (Erulkar et al., 1981; Watson and Kelley, 1989). We sought to determine specifically whether motor neurons in these nuclei express AR mRNA and could thus directly participate in androgen-evoked responses.

Adult male X. laevis have more axons in N. IX–X than do females (Kelley and Dennison, 1990). The larger number of axons observed in male tadpoles requires androgen exposure (Robertson et al., 1994). In juvenile females, testis implantation increases the number of laryngeal nerve axons to male levels (Watson et al., 1993). Although the mechanism by which changes in axon number is achieved is still unclear, these results suggest that motor neurons in N. IX–X of juveniles are sensitive to androgens. Because androgen regulates the expression of its own receptor in the rat brain and prostate and the Xenopus larynx (Menard and Harlan, 1993; Takeda et al., 1991; Fischer et al., 1995), we explored the effect of endocrine manipulations on AR mRNA expression in several CNS regions, including N. IX–X. Our results suggest that AR expression in N. IX–X of juveniles is upregulated by androgens, a process that could facilitate responses induced by androgens in motor neurons.

MATERIALS AND METHODS

Animals and Hormone Treatments

Juvenile frogs (stage PM1, 3 months after metamorphosis is complete; Tobias et al., 1991) were used in the present study. Animals were separated into male and female groups that were either left intact or gonadectomized. Within the gonadectomized group, frogs were either implanted with an empty Silastic tube (Dow Corning, VST 030065) or with a tube containing 5 mg of 5α-17β-ol-3-one androstan (DHT, Sigma). An additional group of gonadectomized females received a Silastic tube containing 5 mg of 17β-estradiol (Sigma). One month after gonadectomy and Silastic tube implantation, animals were intracardially perfused with 5 mL of 0.6% NaCl followed by 10 mL of 4% paraformaldehyde in 1× PBS (2.6 m M KCl, 1.4 m M KH2PO4, 136 m M NaCl, 8 m M NaHPO4, pH 7.2). The brain and spinal cord (CNS) were removed and incubated for 2 h in fixative followed by 20% sucrose in 1× PBS for 4–12 h. Twenty-micron horizontal sections of the brain were cut in a cryostat, thaw mounted onto Superfrost plus microscope slides (Fisher), and stored at −70°C until use.

Northern Blotting

For Northern blot hybridization, an 843-base pair (bp) fragment of the AR, extending from the first zinc finger of the DNA binding domain into the ligand binding domain, was used [Fig. 1(A); He et al., 1990]. This fragment was polymerase chain reaction (PCR) amplified from PM1 and PM3 laryngeal muscle cDNA using primers containing the SP6 (sense) and T7 (antisense) promoters and then gel purified. The fragment was labeled with [32P]-dCTP using the random primer extension reaction. Total RNA was isolated from the brain and spinal cord of 10 PM1 males or 10 PM1 females by the RNazol method (Tel-Test, Inc.) and denatured using glyoxal. Twenty micrograms of total RNA was electrophoretically separated on a 1.2% agarose gel and blotted onto GeneScreen membranes (DuPont) by standard methods. Hybridization was carried out overnight at 42°C under high stringency conditions. Blots were washed in 2× standard saline citrate (SSC), 0.1% SDS at room temperature for 1 h and 0.1× SSC, 0.1% SDS at 65°C for 1 h.

Nuclease Protection Assays

A 495-bp fragment was PCR amplified using the primers Sp6-5′GTATTTCCACGCTTTGAGC3′ and T7-5′GTCGACGACCTCCAC3′ and the 843-bp AR cDNA described previously as the template [Fig. 1(A)]. The PCR product was inserted into a pCR-II vector and transfected into INV-αF′ cells (Invitrogen). We sequenced this cloned PCR fragment; it encodes a portion of the ligand binding domain of the X. laevis αAR (He et al., 1990). After purification, this DNA was cut with XhoI and used to generate 32P-antisense RNA by in vitro transcription (specific activity 4.7 × 109 cpm/μg). Brains were obtained from gonadectomized and DHT-treated gonadectomized males and females. Total RNA was isolated using Trizol (LifeTech) from two regions: the anterior brain (telencephalon, diencephalon, and mesencephalon) and the region of the brain stem containing N. IX–X [Fig. 2(A)]. RNA concentration was measured by spectrophotometry and compared in
Horseradish Peroxidase (HRP) Histochemistry

One group of intact animals was prepared for HRP histochemistry. The larynx was accessed through an incision in the side of the body under the arm and the flexor carpi radialis muscle was approached by an incision in the skin of the forearm. Frogs received HRP type VI (Sigma, approximately 1 mg) as a crystalline powder containing the respective muscles. Three days later, animals were anesthetized, perfused, and brains treated as before. HRP detection was carried out as described previously (Llewellyn-Smith et al., 1992). Briefly, cryostat sections were preincubated in a solution containing 0.006% tetramethylbenzidine (Sigma) in 1 × PBS for 20 min and developed with 0.005% H₂O₂. Sections were then incubated in a solution containing 1 mg/mL 3,3′-diaminobenzidine (DAB) and 16 mg/mL CoCl₂. Finally, a brown precipitate was formed when incubated in 0.05% H₂O₂. Sections were then processed for AR in situ hybridization according to the procedure described below.

In Situ Hybridization

The 495-bp PCR product described previously was in vitro transcribed using the corresponding RNA polymerase (Sp6, sense; T7, antisense) in the presence of digoxigenin-dUTP (dig-UTP) according to the manufacturer's protocol (Boehringer Mannheim). Residual DNA was digested with DNase. Sense and antisense probes were purified by chromatography through Nensorb-20 columns (DuPont) and stored at −70°C until hybridization. The probe concentration was assessed on dot blots by comparison to dig-UTP labeled RNA of known concentration (Boehringer Mannheim). Sections of the brain and spinal cord were postfixed for 20 min at room temperature in 4% paraformaldehyde. The slides were washed twice in 1 × PBS for 5 min each, incubated in proteinase K (10 µg/mL) in 0.1 M Tris-HCl, pH 7.5, 10 mM EDTA for 30 min, followed by a 10-min incubation in 0.25% acetic acid anhydride in 0.1 M triethanolamine. Sections were dehydrated in a graded series of ethanol solutions, placed in chloroform for 5 min and 100 and 95% ethanol for 1 min each, and finally air dried. The AR probe was diluted in hybridization solution (50% formamide, 0.6 M NaCl, 0.06 M sodium citrate, pH 7, 1× Denhardt’s solution, 0.5 mg/mL yeast RNA, 0.2 µg/mL salmon sperm DNA, 10% dextran sulfate) to a final concentration of 2 pg/µL and applied to sections. Slides were covered with parafilm and incubated in a humid chamber at 50°C overnight. The following day, sections were washed with 2 × SSC (0.3 M NaCl, 0.03 M Na citrate, pH 7) and 1 × SSC for 1 h each, followed by 0.5 × SSC for 1 h at 50°C and RNAse A (10 µg/mL) in RNAse buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 0.5 M NaCl) for 30 min at 37°C. Probe concentrations of 10, 2, and 0.1 pg/µL in hybridization solution were assayed to determine the saturating concentration at ~12 h
Figure 2  (A) Diagram of Xenopus laevis brain. Two regions were surveyed for AR mRNA expression by nuclease protection assays: the anterior brain (AB) and brain stem region containing N. IX–X (IX–X). (B) Sensitivity of the nuclease protection assay. Signals due to the protected fragment increase linearly with increasing amounts of total RNA (2.5, 5, and 10 μg) in the assay. (C) Nuclease protection assay comparing expression of AR mRNA in IX–X and AB. Optical density analysis reveals that expression in the brain stem is ~58% higher than in the anterior brain. Insert: Total RNA from the brain stem and anterior brain analyzed in 1% agarose gel. (B, C) MW lane: RNA molecular weight markers; arrowheads, 562 and 363 nt. P lane: Undigested AR probe. tRNA lane: As an internal control, the AR probe was hybridized to tRNA. Occasionally, a band of undigested probe was seen in the tRNA lane and in the 2.5 and 10 μg lanes.

(overnight) of hybridization [Fig. 3(A–C)]. To control for specificity, hybridization signals with a 843 nucleotide (nt) dig-labeled probe (similar to that used for Northern blot) were compared to signals with the 495 nt probe; similar results were obtained (data not shown). In addition, one group of sections was incubated with similar concentrations of dig-labeled sense riboprobe [Fig. 3(D)]. To control for nonspecific signals another set of sections was preincubated with 20 μg/mL RNAs A for 30 min at 37°C [Fig. 3(E)]. The immunohisto

Figure 3  Nonisotopic hybridization signals in N. IX–X in control experiments. The tissue was hybridized with (A) 10 pg/μL, (B) 2 pg/μL, and (C) 0.1 pg/μL of probe in the hybridization solution. No hybridization signals were found in sections hybridized with (D) sense probe or pretreated with RNase A. Scale bar (E) = 50 μm.
chemical procedure for recognizing dig-labeled probes was as previously described (Pérez and Hoyer, 1995). Nuclei labeled with the AR probe were identified according to Simpson et al. (1986).

**Cell Quantification and Statistical Analysis**

Cells expressing AR mRNA were recognizable by blue staining in the cytoplasm. The number of AR expressing cells in N. IX-X was determined in consecutive horizontal sections at 250× using a light microscope. Only cells with a visible nucleus were counted. Statistical comparison of the experimental groups was performed using analysis of variance (ANOVA) with the post hoc Newman–Keuls test.

**RESULTS**

**Northern Blot Analysis**

Because two forms of the AR mRNA have been reported in the *Xenopus* larynx, we first determined if these mRNA forms are expressed in the CNS. A probe that recognizes the two isoforms of the *X. laevis* AR, ARα and ARβ, was used to identify transcripts expressed in the CNS [Fig. 1(A)]. The ARα, which is a 9.6 kb mRNA, is the only transcript expressed in both male and female CNS at PM1 [Fig. 1(B)].

**Expression of AR mRNA in *X. laevis* Brain**

Nuclease protection assay produced a single protected fragment, demonstrating the specificity of the probe used for this assay and in situ hybridization [Figs. 1(A), 2(B,C)]. To determine the sensitivity of the nuclease protection assay, increasing concentrations (2.5, 5, and 10 μg) of total RNA from the anterior brain were hybridized to the AR probe [Fig. 2(B)]. The optical density values of the respective protected fragments were 18,800, 34,831, and 78,550 units and are thus linearly related to RNA content in the assay. In the first assay in the females, the optical density value of the protected fragment representing AR mRNA was 72% higher in the brain stem than in the anterior brain (optical density values in the brain stem = 51,267 vs. anterior brain = 14,353). This result was confirmed in a second assay [Fig. 2(C)]; the optical densities of the protected fragment were anterior brain 25,195 and brain stem 45,881 (45% higher). These results suggest that levels of AR mRNA are higher in the brain stem than in the anterior brain.

**Localization of AR mRNA Expression in *X. laevis* CNS**

AR probe concentrations of 10, 2, and 0.1 pg/μL hybridization solution were compared to determine the optimum concentration for *in situ* hybridization [Fig. 3(A–C)]. A concentration of 2 pg/μL hybridization solution was used for quantification of the number of AR mRNA expressing cells because this concentration saturated the hybridization reaction [Fig. 3(B)] without the increase in background observed at the higher concentration [Fig. 3(A)]. No hybridization signal was observed in sections incubated with dig-labeled sense probe [Fig. 3(D)] or preincubated with RNase A [Fig. 3(E)].

No cells in telencephalon, diencephalon, or mesencephalon showed high levels of hybridization to

*Figure 4* Hybridization signals in *X. laevis* CNS. Horizontal sections of the brain are shown from (A) rostral to (F) caudal. In each figure, up is anterior and down is posterior. Cells labeled with the AR probe display a blue cytoplasmic signal. Brain stem nuclei were identified according to Simpson et al. (1986). (A) Weak hybridization signals were found in the striatum (St), preoptic area (Pa), and thalamus (Th). (B) Hybridization signals were observed in the vestibular (Ve) and lateral line (LNL) nuclei; the AR probe did not hybridize to granule cells of the cerebellum (Cer). (C) Hybridization signals in cells of the nucleus isthmus (Is) and dorsal tegmental nucleus of the medulla (Dr). No hybridization signals with the AR probe were found in the central grey (Cg). (D) Hybridization signals in the trigeminal (V) and facial (VII) motor nuclei. (E) *In situ* hybridization signals in the motor nuclei of cranial nerves IX–X (IX–X) and reticular formation (Re). (F) Motor neuron columns in the anterior spinal cord display high AR hybridization signals. (G) Cells in the postotic or acoustic ganglion were strongly labeled with AR probe. (H) Section at the level of the spinal cord: no hybridization signals were apparent in sections hybridized with sense probe. Original magnifications in (A–F) and (H) are as in (A). Scale bar = (A) 100 μm, (G) 25 μm.
the AR probe. Cells with light or moderate label in the cytoplasm following hybridization with the AR probe were found in the dorsolateral striatum of the telencephalon [Fig. 4(A)], thalamus [Fig. 4(A)], magnocellular nucleus of the preoptic area [Fig. 4(A)], anterior hypothalamus (not shown), and mesencephalic nucleus of the trigeminal nerve (not shown). In contrast, cells in the anterior preoptic nucleus, anterior thalamus, and optic tectum were not labeled with the AR probe (not shown).

Identification of brain stem nuclei follows the atlas and terminology of Simpson et al. (1986). In the brain stem, cells in the nucleus of the lateral line nerve [Fig. 4(B)], the vestibular nucleus [Fig. 4(B)], nucleus isthmus [Fig. 4(C)], and motor nuclei of cranial nerves V and VII [Fig. 4(D)] displayed moderate hybridization signals. Cells of the interpeduncular nucleus were also moderately labeled (not shown). A subpopulation of cells within the dorsal tegmental area of the anterior medulla [Fig. 4(C)] displayed moderate to high hybridization. No hybridization was observed in the granular layer of the cerebellum or within the central grey [Fig. 4(B,C)].

The strongest hybridization signals to the AR probe in the CNS were found in cells of N. IX–X [Fig. 4(E)] and the motor column of the spinal cord [Fig. 4(F)]. Cells of the reticular formation displayed low to moderate hybridization signals [Fig. 4(E)]. In the peripheral nervous system, cells of the postotic ganglia displayed a strong in situ hybridization signal with the AR probe [Fig. 4(G)]. No hybridization signal was seen when the sense probe was used [Fig. 4(H)].

HRP-labeled neurons in the N. IX–X [Fig. 5(A)] and spinal cord at the level of the second and third spinal nerves [Fig. 5(B)] express AR mRNA. In N. IX–X, we also found HRP-labeled motor neurons with no hybridization to the AR probe and HRP-negative cells that express AR mRNA [Fig. 5(A)]. Double-labeled cells were observed in N. IX–X of both sexes. The number of HRP-labeled cells was smaller than the number of N. IX–X motor neurons expected for PM1 (Robertson et al., 1994), suggesting that the HRP injection did not label all N. IX–X motor neurons.

**Hormonal Regulation of Number of AR mRNA Expressing Cells in N. IX–X**

Gonadectomy and DHT treatment both had a marked effect on the number of AR expressing cells in N. IX–X. An increase in AR mRNA signals within N. IX–X cells was observed in DHT treated gonadectomized males and females as compared to untreated gonadectomized animals (Fig. 6). A two-way ANOVA comparing intact, gonadectomized, and DHT treated males and females revealed significant effects of treatment ($F = 17.93$, df = 2, $p < 0.0001$) and sex ($F = 11.35$, df = 1, $p < 0.01$). This analysis was followed by a one-way ANOVA with these six groups of animals ($F = 9.55$, df = 5, $p < 0.0001$) and a Newman–Keuls post hoc analysis for comparisons. In intact animals, the number of AR mRNA positive cells in N. IX–X of males was significantly higher than in females (Newman–Keuls, $p < 0.05$; Fig. 7). In contrast, the number of AR expressing cells was not significantly different between sexes in either untreated or DHT treated gonadectomized groups (Fig. 7). One month after gonadectomy, the number of AR expressing cells in N. IX–X was significantly reduced in males, but not in females, compared to intact animals (Newman–Keuls, $p < 0.05$). Gonadectomized males and females treated with DHT had significantly more AR mRNA positive cells than the respective untreated gonadectomized animals (for both comparisons, Newman–Keuls, $p < 0.01$). The effect of estradiol treatment was independently analyzed in females using a one-way ANOVA ($F = 10.7$, df = 3, $p < 0.008$). The number of AR expressing cells in gonadectomized females treated with estradiol was significantly smaller than in DHT treated gonadectomized females (Newman–Keuls, $p < 0.05$) but did not significantly differ from intact or gonadectomized females ($p > 0.05$; Fig. 7).

**Regulation of AR mRNA Expression in Anterior Brain**

The effect of DHT on AR mRNA content in the anterior brain was analyzed by nuclease protection assay. In a first set of experiments 5 μg of total RNA from eight to nine animals per group was analyzed (Fig. 8). Optical density values for the male groups were: gonadectomized = 25,152 and gonadectomized-DHT treated = 73,461, and for the female groups: gonadectomized = 16,174 and gonadectomized-DHT treated = 19,052. Thus nuclease protection assays revealed that AR mRNA levels in the anterior brain were 66% higher in DHT-treated gonadectomized males and 15% higher in DHT-treated gonadectomized females. To confirm these results, total RNA was extracted from a second set of animals (12–18 animals per group) and analyzed as above. Optical density values for the male groups were: gonadectomized = 19,427, gonadectomized-DHT
treated = 24,531 (20.8% increase), and for the female groups were gonadectomized = 14,353, gonadectomized-DHT treated = 26,186 (45% higher). We conclude that DHT treatment increases AR mRNA levels in the anterior brain of both gonadectomized males and females.

DISCUSSION

Motor Neurons in N. IX-X Express AR mRNA

Target cells whose functions are directly influenced by steroid hormones require the expression of spe-

Figure 5 (A) Motor neurons in N. IX-X and (B) motor neurons that project to the arm were labeled with HRP (brown reaction) and AR probe (blue reaction). Some N. IX-X motor neurons express AR mRNA (arrowheads) whereas others do not (arrows). Scale bar = (A) 30 \mu m, (B) 50 \mu m.

Figure 6 AR mRNA expressing cells of N. IX-X in (A, C) gonadectomized and (B, D) gonadectomized/DHT treated (A, B) males and (C, D) females. IX-X, nucleus IX-X. An increase in hybridization signal was observed in cells of gonadectomized/DHT treated groups as compared to untreated/gonadectomized animals. Scale bar = 100 \mu m.
Androgen receptor mRNA expressing cells in N. IX-X of males (striped bars) and females (white bars). Mean values ± S.E.M. are presented; number of animals analyzed are given with each bar. Intact females have significantly fewer AR mRNA expressing cells than males (Newmann-Keuls, p < 0.05). Gonadectomized (GX) males have significantly fewer AR mRNA expressing cells than intact males (Newman-Keuls, p < 0.05). Gonadectomized and DHT treated (GX-DHT) males and females have significantly more AR mRNA expressing cells than untreated gonadectomized animals (Newman-Keuls, p < 0.01). Gonadectomized females treated with estradiol (GX-E2) have significantly fewer AR mRNA expressing cells than DHT treated females (Newman-Keuls, p < 0.05).

Specific receptors. Once steroid receptors are ligand activated, they act as transcription factors controlling the expression of hormone-regulated genes. Two neuromuscular systems in X. laevis have been shown to be regulated by androgens: one that controls reproductive vocalizations and another involved in the control of clamping during amplexus (reviewed in Kelley, 1996). The muscles in these systems express high levels of AR as demonstrated by binding assays, immunocytochemistry, and in situ hybridization for AR mRNA (Erulkar et al., 1981; Fischer et al., 1993, Dorlöchter et al., 1994). AR binding was also demonstrated in motor nuclei of the CNS of X. laevis (Kelley et al., 1975; Kelley, 1981; Erulkar et al., 1981; Gorlick and Kelley, 1986; Brennan and Henderson, 1995).

While it seemed very likely that some, if not all, of the cells that express AR in motor nuclei of laryngeal and arm nerves were motor neurons (Kelley, 1980; Erulkar et al., 1981), this identification had not been made directly. The specification of cell types expressing AR in mRNA in N. IX-X and anterior spinal cord was approached in the present study by analyzing in situ hybridization signals in cells back labeled from laryngeal and arm muscles with HRP. Results of the present study indicate that motor neurons in the medulla that project to laryngeal muscle and motor neurons in anterior spinal cord that project to the flexor carpi radialis muscle of the arm express AR mRNA. In addition, AR mRNA was not detected in a subpopulation of motor neurons. Thus, in intact males and females, a population of laryngeal motor neurons may not express AR mRNA. N. IX-X contains about twice as many cells as there are laryngeal axons (Kelley and Dennison, 1990), and the only motor neurons in the nucleus are those projecting to the larynx (Simpson et al., 1986). Backfills with DiI demonstrated the presence of interneurons in N. IX-X that are sexually dimorphic in dendritic extent (Kelley et al., 1988; Watson and Kelley, 1989). Because not all motor neurons were HRP filled in the present study (i.e., the number of HRP-labeled cells was smaller than the number of laryngeal motor neurons expected at PM1; Robertson et al., 1994) and because we do not have spe-
specific markers for interneurons, we cannot yet determine whether interneurons in N. IX–X express AR mRNA. However, we can conclude that in intact juvenile male and females the population of laryngeal motor neurons is mixed with respect to AR mRNA expression.

**AR mRNA Widely Expressed in X. laevis CNS**

*In situ* hybridization signals and nuclease protection assays demonstrated that there is wide expression of AR mRNA in the CNS. Levels of transcript are higher in the brain stem than in anterior regions of the brain. The *in situ* hybridization signals found in the cytoplasm of cells in the motor column of the spinal cord and in cells of motor nuclei of the brain stem were comparatively higher than signals in cells of other nuclei of the CNS. Previous to this study, the distribution of AR binding in the *X. laevis* CNS was known from steroid autoradiographic examination using radioactive testosterone and DHT (Kelley et al., 1975; Kelley, 1980, 1981; Gorlick and Kelley, 1986). Binding of radioactively labeled hormone is more restricted than AR mRNA expression. Cells of the striatum, posterior preoptic area, motor nuclei V and VII, nucleus isthmi, and interpeduncular nucleus do not concentrate testosterone or DHT but do express AR mRNA. On the other hand, AR mRNA expression and ligand binding correlate in the ventral thalamus, the dorsal tegmental nucleus, N. IX–X, reticular formation, and spinal cord. Both methods reveal the absence of AR expression in the anterior preoptic nucleus, anterior thalamus, granular layer of the cerebellum, and within the central grey. AR mRNA expression in the postotic (acoustic) ganglion agrees with observations of DHT binding (D. Kelley, unpubl. observ.). Androgens may contribute to auditory sensitivity (Kelley, 1980).

A broad distribution of AR mRNA was also described in the rat brain, including cortex and cerebellum that do not concentrate significant amounts of radioactive androgens (Simerly et al., 1990; Osada et al., 1993). The probe used in this study was directed against the ligand-binding domain of the receptor. This region is highly specific for each steroid receptor, and it is thus unlikely that the pattern of hybridization is the result of cross hybridization to other receptors. Further, specificity of the probe is demonstrated in the nuclease protection assay as a single band of the appropriate molecular weight.

Differences between autoradiography and *in situ* hybridization might be due to differences in the sensitivity of the assays or to unrelated ratios of AR mRNA expression to translation. For example, some neurons may not express sufficient AR mRNA for efficient translation. We attempted to address this question using the PG-21 antibody, a polyclonal antiserum raised against the first 21 amino acids of the rat and human AR at the amino terminus (Prins et al., 1991). Cell nuclei of HRP-labeled motor neurons were immunoreactive to the antibody. However, in Western analysis of proteins obtained from *X. laevis* spinal cord, the antibody recognized two protein bands: one at approximately 100 kDa, probably corresponding to the AR, and another at 170 kDa, of unknown identity. Because the PG-21 antibody recognizes multiple bands, analysis of AR mRNA and AR protein expression in the CNS of *X. laevis* awaits development of more specific reagents.

One AR transcript is present in juvenile *X. laevis* CNS. This result contrasts with the two isoforms expressed in the rat brain (Burgess and Handa, 1993). We previously described two AR mRNA isoforms (9.6 and 8 kb; Fischer et al., 1993) expressed in the larynx of juvenile *X. laevis*; interestingly, the shorter transcript is related to androgen-evoked induction of laryngeal cell proliferation (Fischer et al., 1995). We do not yet know whether this shorter transcript is expressed earlier in the development of the CNS when cell proliferation is prevalent (Gorlick and Kelley, 1987).

As in binding experiments, *in situ* hybridization and nuclease protection assays suggest that motor neuron nuclei are the major sites of AR expression in the *X. laevis* CNS. The wide distribution of AR mRNA suggests that cells in different regions of the CNS have the potential to express the protein and that androgens might participate in a variety of CNS functions.

**Sexual Dimorphism in AR mRNA Expression in N. IX–X**

The number of AR mRNA expressing cells in N. IX–X is higher in males than in females. It is possible that this characteristic may also occur in sexually dimorphic nuclei of other vertebrates (for a review see Arnold and Gorski, 1984). At least two mechanisms may explain sex differences in the number of AR mRNA expressing cells in the CNS. First, cell division and/or death of this population may differ in the sexes. Second, the expression of AR mRNA may be under the control of hormones whose circulating levels differ in the sexes. These
explanations are not mutually exclusive, and the second may serve as a mechanism for the first. While no sex difference in cell proliferation is apparent in N. IX–X of X. laevis, an augmented decrease in the number of axons in the laryngeal nerve of females during tadpole stages leads to a sexually dimorphic axonal content in juveniles and adults (Gorlick and Kelley, 1987; Kelley and Dennis, 1990). During tadpole stages, axonal decreases can be prevented by androgens (Robertson et al., 1994). Superficially, these results appear to parallel the control of motor neuron numbers in the SNB nucleus of the rat spinal cord, where androgen in males prevents ontogenetic cell death (Nordeen et al., 1985). Thus, androgen attenuation of cell death in N. IX–X is a mechanism that may explain differences in the number of cells. It would be of interest to determine whether AR expressing motor neurons are preferentially maintained by hormones during naturally occurring cell death.

**Hormonal Regulation of AR mRNA Expression in N. IX–X**

In these juveniles, endocrine manipulations had powerful effects on the number of AR mRNA expressing cells in N. IX–X. Decreases in AR mRNA expressing cells were found in gonadectomized animals, and DHT treatment reversed this effect. The increase in AR mRNA expressing cells was hormone specific because estradiol, a metabolite of testosterone, did not have a significant effect. The effect of gonadectomy, or androgen removal, is also marked in these animals because they have fewer AR mRNA expressing cells than intact animals. At the moment we cannot determine if the increased number of AR mRNA expressing cells in males represents more expression per cell or more cells in the nucleus due to reduced cell death. Because cells in N. IX–X are extremely sensitive to circulating androgen levels, the sexual dimorphism in the number of AR mRNA expressing cells in N. IX–X at PM1 could be due simply to sex differences in circulating levels of androgens. However, at PM1 there are no sex differences in DHT or testosterone levels; levels in males are not significantly higher than those in females until PM4, some 6 months later (Kang et al., 1995). Regardless, the expression of the receptor is susceptible to variations in circulating levels of androgens in juvenile animals.

Hormonal regulation of AR expression differs in different tissues and species. Our data are consistent with decreases in AR immunoreactivity in the rat brain following long-term gonadectomy and recovery of AR expression due to steroid treatments (Menard and Harlan, 1993; Sar et al., 1990). Similarly, in cultured insect cells infected with a baculovirus containing an AR cDNA, upregulation of AR expression can be induced by short exposure to testosterone (Kallio et al., 1994). However, in the rat brain Burgess and Handa (1993) observed upregulation of AR mRNA expression by long-term gonadectomy and downregulation following short-term treatments with DHT. In the PM1 or PM2 X. laevis larynx, androgen treatment downregulates AR mRNA expression; the kidney, however, displays increases in AR mRNA content (Fischer et al., 1995).

We can conclude that the expression and regulation of AR mRNA are highly specific to tissue, developmental stage, and hormone treatment. The functional consequences of this regulation are as yet unclear. However, because androgen treatment in juvenile animals causes marked increases in the number of axons in the IX–X nerve, it is likely that this effect requires an increase in AR expression in motor neurons, as opposed to a decrease, to achieve hormone-evoked induction of transcription related to these complex structural changes.

As in other species including humans, AR mRNA expression in the CNS of X. laevis is higher in the motor nuclei and spinal cord than other brain regions. There is a sexual dimorphism in the number of AR mRNA expressing cells in N. IX–X. The transcription of the receptor can be altered in this nucleus either by absence of endogenous agonist or hormone replacement. Our data suggest that motor neurons in this nucleus are direct targets for androgen, and that regulation of AR mRNA expression could play an important role in the involvement of these cells in sexually differentiated behaviors.

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**REFERENCES**


ROBERTSON, J. C., WATSON, J. T., and KELLEY, D. B. (1994). Androgen directs sexual differentiation of la-
