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Distribution of Androgen Receptor mRNA Expression in Vocal, Auditory, and Neuroendocrine Circuits in a Teleost Fish

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Abstract

Across all major vertebrate groups, androgen receptors (ARs) have been identified in neural circuits that shape reproductive-related behaviors, including vocalization. The vocal control network of teleost fishes presents an archetypal example of how a vertebrate nervous system produces social, context-dependent sounds. We cloned a partial cDNA of AR that was used to generate specific probes to localize AR expression throughout the central nervous system of the vocal plainfin midshipman fish (*Porichthys notatus*). In the forebrain, AR mRNA is abundant in proposed homologs of the mammalian striatum and amygdala, and in anterior and posterior parvocellular and magnocellular nuclei of the preoptic area, nucleus preglomerulosus, and posterior, ventral and anterior tuberal nuclei of the hypothalamus. Many of these nuclei are part of the known vocal and auditory circuitry in midshipman. The midbrain periaqueductal gray, an essential link between forebrain and hindbrain vocal circuitry, and the lateral line recipient nucleus medialis in the rostral hindbrain also express abundant AR mRNA. In the caudal hindbrain-spinal vocal circuit, high AR mRNA is found in the vocal prepacemaker nucleus and along the dorsal periphery of the vocal motor nucleus congruent with the known pattern of expression of aromatase-containing glial cells. Additionally, abundant AR mRNA expression is shown for the first time in the inner ear of a vertebrate. The distribution of AR mRNA strongly supports the role of androgens as modulators of behaviorally defined vocal, auditory, and neuroendocrine circuits in teleost fish and vertebrates in general.

Keywords

vocal control system; hearing; inner ear; hypothalamus; estrogen receptor; aromatase

Androgen receptors (ARs) and androgen-concentrating cells have been consistently identified in neural circuits that shape reproductive-related behaviors, including sexually dimorphic nuclei that control vocalization, across most vertebrate taxa: amphibians (Kelley, 1986; Perez et al., 1996), reptiles (Tang et al., 2001), and birds (Gahr, 2001; Ball et al., 2002; Schlinger and Brenowitz, 2002; Kim et al., 2004) (for review, see Bass, 2008). While androgen-concentrating neurons have long been known in the central nervous system (CNS) of fish as well (e.g., Morrell et al., 1975; Fine et al., 1996), recent studies have shown that androgens can strongly modulate the vocal pattern generating circuitry in teleost fish (Remage-Healey and Bass, 2004, 2006a, 2007), although the loci for possible direct androgen actions on the vocal system remain unknown. The current study delineates the position of ARs in the plainfin midshipman fish, *Porichthys notatus*, that has become a well-established model system for

studying the neural and hormonal mechanisms of vocal-acoustic communication among vertebrates (Bass and McKibben, 2003).

The plainfin midshipman has two male morphs that exhibit alternative reproductive tactics including divergent vocal behaviors (Brantley and Bass, 1994; Lee and Bass, 2006). Territorial, type I males defend nests and court females acoustically with territorial and advertisement calls generated via the contraction of paired muscles attached to the gas-filled swim bladder (Cohen and Winn, 1967; Bass, 1996). Unlike type I males, type II males are nonterritorial, reach sexual maturity early, and invest in large testes size rather than body size and vocal muscle, and either “sneak” or “satellite” spawn in competition with type I males to fertilize eggs of which the type I will invest in parental behavior (Brantley and Bass, 1994; Bass, 1996). In general, type II males and females are similar to each other but differ from type I males in a suite of somatic, endocrinological, and neurological traits, including differing levels of circulating androgens (Bass, 1996). Sexual polymorphisms for this species are best exemplified in the vocal motor system where a hindbrain-spinal vocal pattern generator and sound-producing muscle is hypertrophied in type I males (Bass and Marchaterre, 1989; Bass et al., 1996). The capacity for multiple types of vocalizations (advertisement and agonistic calls) of widely divergent duration (msec to >1 hour) is reflected in the expanded vocal motor system in type I males, whereas females and type II males are limited to producing only short duration (msec) agonistic calls (Brantley and Bass, 1994).

The vocal and auditory systems in midshipman and the closely related toadfish (same family: Batrachoididae; Nelson, 2006) show a conserved pattern of organization that shares many traits with tetrapods (Bass and Baker, 1997; Goodson and Bass, 2002; Bass et al., 2005, 2008; Kittelberger et al., 2006). An expansive vocal motor system (Fig. 1) includes a midline motor nucleus (VMN) that spans the caudal hindbrain-rostral spinal cord boundary and ipsilaterally innervates each vocal swim bladder muscle via paired occipital nerve roots (Bass and Baker, 1990; Bass et al., 1994). Each VMN receives bilateral input from adjacent columns of vocal pacemaker neurons (VPN) that, in turn, are innervated by a more rostral prepacemaker nucleus (VPP, formerly ventral medullary nucleus). Neurophysiological studies support the hypothesis that the VPP-VPN-VMN circuit collectively comprises a vocal pattern generator (VPG) that directly determines the temporal pattern of natural calls (Bass and Baker, 1990; Ramage-Healey and Bass, 2004; Chagnaud et al., 2009) and compares closely to vocal patterning circuits in tetrapods (Bass et al., 2008). Other studies show that auditory inputs interface with vocal nuclei in the forebrain, midbrain, and hindbrain (Bass et al., 2000; 2001; Goodson and Bass, 2002; Kittelberger et al., 2006).

Midshipman fish and toadfish are ideal model systems to investigate the effects of androgens on defined neural circuitry which directly impacts vocal-acoustic communication during social behavior. Androgens masculinize vocal motor neurons and muscle (reviewed in Bass and Forlano, 2008) and rapidly modulate vocal patterning (reviewed in Ramage-Healey and Bass, 2006a; Bass and Ramage-Healey, 2008). Androgens are elevated during nesting and gonadal recrudescence in males and females (Brantley et al., 1993a; Knapp et al., 1999; Modesto and Canario, 2003; Sisneros et al., 2004a; Fine et al., 2004), during advertisement calling in males (Knapp et al., 2001), and during acoustic playback challenge to nesting males (Ramage-Healey and Bass, 2005). Androgens also induce changes in vocal behavior in nesting males in their natural habitat (Ramage-Healey and Bass, 2006b). Seasonal peaks in circulating androgens correlate with high brain aromatase (estrogen synthase) mRNA expression (Forlano and Bass, 2005a) in both males and females. Testosterone upregulates aromatase expression in glial cells throughout the brain (Forlano and Bass, 2005b; Bass and Forlano, 2008) and induces natural, seasonal-like changes in the temporal encoding of the frequency content of male calls in the peripheral auditory system (Sisneros et al., 2004b).

The main goal of this study was to test the hypothesis that central integration sites for vocal, auditory, and neuroendocrine functions in the midshipman fish would show high levels of AR expression, consistent with the known role of androgens in modulating vocal-acoustic and reproductive behaviors.

MATERIALS AND METHODS

Animals

Midshipman fish were collected from field sites in northern California during the spawning season or in Monterey Bay, CA at other times of the year and maintained in seawater tanks until sacrificed shortly afterwards (see Sisneros et al., 2004a, for a description of the reproductive and nonreproductive time periods). The animals chosen for study were from the spring and summer months when plasma levels of steroids in general are elevated in females and males (Sisneros et al., 2004a). All experimental protocols were approved by the Cornell University Institutional Animal Care and Use Committee.

Cloning of partial cDNA of midshipman AR

Methods were previously reported for cloning of partial cDNA for midshipman ER α (Forlano et al., 2005) and were similar to those used to clone a partial cDNA for the aromatase gene from this species (Forlano et al., 2001). Degenerate primers (forward: GARGCNTCNGGNTGYCAYTAYGG; reverse: NCCYTTNGCCCAYTTNACNACYTT) were designed based on highly conserved regions in the DNA and ligand binding domains of six AR sequences from four teleost species (*Halichoeres trimaculatus*, *Oncorhynchus mykiss* [alpha and beta isoforms], *Anguilla japonica* [alpha and beta isoforms], and *Chrysophrys major*). Partial cDNA clones (462 bp) of AR were amplified from vocal muscle, brain, and the sensory epithelium from the saccule division of the inner ear of two type I males in reproductive condition.

Reverse-transcription polymerase chain reaction (RT-PCR)

Internal, midshipman-specific primers (forward: GCTGGAATGACCCCTTGGAGCA; reverse: TCAACTCCCACGTGGTCTTCCTC) were employed to identify AR mRNA transcripts (150 bp) from brain, sonic muscle, and saccular epithelium of three type I males, two females and a type II male. Brains were dissected into four areas: 1) a large forebrain region including the olfactory bulbs, telencephalon, and preoptic area; 2) a middle region including the caudal diencephalon, midbrain tectum, and tegmentum; 3) the corpus of the cerebellum; and 4) remaining hindbrain and rostral spinal cord. Total RNA was isolated from various tissues and DNase-treated (Ambion, Austin, TX) according to the manufacturer's instructions, after which 2.5 μ g of RNA was reverse-transcribed as previously reported (Forlano et al., 2001). PCR was performed in a final volume of 50 μ L containing 1 $\times$ reaction buffer, 1.5 mM MgCl₂, 200 μ M each dNTP, 0.5 μ M each primer, 0.5 μ L Tfi DNA polymerase, and 1 μ L of first-strand cDNA reaction. The reaction was run under the following conditions: 94°C for 4 minutes followed by 35 cycles of 94°C for 45 seconds, 56°C for 45 seconds, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. Negative controls included a no template control, and samples were verified as negative for genomic DNA after DNase treatment. Reactions were run on a 2% agarose gel stained with ethidium bromide to detect the presence of transcripts in the various tissues.

In situ hybridization (ISH)

The procedure for ISH detection of AR mRNA was similar to our previous study for ER α mRNA (Forlano et al., 2005). Adult midshipman fish were deeply anesthetized in 0.025% benzocaine (Sigma Chemical, St. Louis, MO) in seawater and perfused transcardially with

teleost ringers followed by 4% paraformaldehyde in 0.1M phosphate buffer (PB; pH 7.2). Brains (five type I males, three type II males, three females) and saccular epithelium of the inner ear (two females) were removed and postfixed in the same fixative for 1 hour and stored in PB. Prior to sectioning, various tissues were cryoprotected overnight in 30% sucrose in PB and frozen in Cryo-M-bed (Hacker Instruments, Huntington, UK). Sections were made at 30 μ m in the transverse plane for brain tissue and at 20 μ m in the oblique plane for the inner ear; alternate sections were collected onto Super-frost Plus slides (Erie Scientific, Portsmouth, NH) and stored at -80°C until processed. Prior to hybridization, slides were equilibrated to room temperature for 10 minutes, washed $2 \times$ in 0.1 M potassium phosphate-buffered saline (KPBS; pH 7.2) and placed into freshly prepared 0.25% acetic anhydride in 0.1M triethanolamine $2 \times$ 5 minutes, dehydrated in a series of ethanol washes and chloroform, and air-dried.

Hybridization was carried out with a mixture of three oligo probes antisense to nucleotides 160–186 (AAATGTTTTGAAGCTGGAATGACCC), 240–265 (CCTCTCCAGGAGTCTGTTGAGATCA), 401–425 (CTCTGCTCACCAGCCTCAATGAGC) of the 462-bp sequence of midshipman AR. A mixture of sense oligos from which the antisense sequences were derived were used as negative controls. Oligonucleotides were labeled with a terminal deoxynucleotidyl transferase reaction using α - ^{32}P d-ATP (SA, 1,000–3,000 Ci/mmol; NEN, Boston, MA). Hybridization solution ($4 \times$ SSC [$1 \times$ SSC = 0.15 M sodium chloride; 0.015 M sodium citrate, pH 7.2], 40% deionized formamide, 500 $\mu\text{g}/\text{mL}$ denatured calf thymus DNA, 250 $\mu\text{g}/\text{mL}$ transfer RNA, $4 \times$ Denhardt's solution, 4 mM EDTA, 5 mM sodium phosphate [pH 6.5], 10% [wt/vol] Dextran sulfate and 3×10^6 cpm total radiolabeled probe [1.0×10^6 cpm each probe]) was placed on each slide (300 μL), coverslipped with parafilm, and incubated overnight (at least 15 hours) in a humidified 37°C chamber. Following hybridization, slides were briefly washed twice at 23°C in $1 \times$ SSC, washed twice for 30 minutes at 55°C in $1 \times$ SSC in a shaking water bath, and washed once in $1 \times$ SSC in 0.1% Triton X-100 at 23°C , briefly rinsed in distilled water, 70% ethanol, and air-dried. Slides were then exposed to Biomax MR film (Eastman Kodak, Rochester, NY) for 3 days at 4°C to confirm signal, and subsequently dipped in nuclear emulsion (NTB, Kodak) and exposed for 4 weeks at 4°C . Slides were developed in Kodak D-19 (4 minutes at 14°C), rinsed in distilled water (10 seconds at 14°C), and fixed (Kodak GBX fixer, 5 minutes at 14°C), rinsed in running distilled water (5 minutes), counter-stained in cresyl violet, dehydrated, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). The neuroanatomical nomenclature of Braford (1995), Braford and Northcutt (1983), and Northcutt (1995) was adopted for this study.

Photomicroscopy

Darkfield optics were used to best visualize the overall mRNA signal pattern throughout the brain at low magnification, while brightfield optics were used to best visualize ISH signal at higher magnification over Nissl-stained tissue. Photographs were taken using Kodak Gold color film (iso 400 for darkfield; iso 100 for brightfield) and a 35 mm Nikon camera mounted on a Nikon ES800 compound microscope. Negatives were then scanned at 1800 dpi, compiled into plates, converted to grayscale, contrast-enhanced, and labeled in Adobe Photoshop CS4 (San Jose, CA).

RESULTS

Cloning of partial cDNA for midshipman AR

A predicted 462-bp sequence was amplified from adult male vocal muscle cDNA and was verified from 12 independent clones, as well as four brain clones, and five clones from the saccular epithelium of the inner ear of males. Midshipman vocal muscle is a known androgen-sensitive target (Brantley et al., 1993b). Figure 2 shows the partial cDNA sequence with

translated amino acid sequence below. The sequence spans conserved regions of the DNA and ligand binding regions of the AR gene across vertebrates. Figure 3 shows a comparison of midshipman AR translated amino acid sequence to other vertebrate ARs. A BLAST (National Center for Biotechnical Information) search using the midshipman translated protein suggests a close match to other teleost AR beta (AR β) subtypes. Multiple clones from different tissues support the likelihood of a single AR gene in midshipman. A sequence alignment revealed an 89% identity to three spot wrasse (*Halichoeres trimaculatus*) AR (GenBank Access. No. AF326200.1), 88% sequence identity to Nile Tilapia (*Oreochromis niloticus*) AR β (GenBank Access. No. AB045212.1), stickleback (*Gasterosteus aculeatus*) AR β 1 and β 2 (GenBank Access. No. AY247206.1 and AY247207.1, respectively) and Atlantic croaker (*Micropogonias undulatus*) AR (GenBank Access. No. AY701761.1), 85% identity to rainbow trout (*Oncorhynchus mykiss*) AR β (GenBank Access. No. AB012096.1), 82% identity to zebrafish (*Danio rerio*) AR (GenBank Access. No. EF440290.1), 84% identity to Japanese eel (*Anguilla japonica*) AR β and 80% to AR α (GenBank Access. No. AB025361.1 and AB023960.1, respectively), 69% to African clawed frog (*Xenopus laevis*) AR α (GenBank Access. No. NM_001090884.1), 68% identity to zebra finch (*Taeniopygia guttata*) AR (GenBank Access. No. NM_001076688.1), 66% to human (*Homo sapiens*) AR (GenBank Access. No. L29496.1), and 65% identity to mouse (*Mus musculus*) AR (GenBank Access. No. NM_013476.3).

RT-PCR

AR transcripts were identified in four CNS regions in adult individuals (Fig. 4): a large forebrain region including the olfactory bulbs, telencephalon, and preoptic area; a middle region including the caudal diencephalon, midbrain tectum, and midbrain tegmentum; the corpus of the cerebellum; and remaining hindbrain and rostral spinal cord. AR mRNA was also found in saccular epithelium of the inner ear and vocal muscle of both adult females and type I males. No transcripts were detected when DNase-treated RNA was used as a template (no RT) and in no template controls for PCR reactions.

In situ hybridization

A neuroanatomical atlas of AR mRNA expression is represented in Figures 6-12 at 13 transverse levels shown in Figure 5 from a type II male brain. For each representative level of the atlas, the left half of the Nissl-stained hybridized section is presented in darkfield (left) at low magnification and then in brightfield (middle). Higher-magnification views of individual nuclei are displayed in brightfield (right) to demonstrate the most abundant AR hybridization patterns. The general patterns of hybridization are representative of all animals used in the study (type I, II males and females). No specific hybridization signal was seen with negative controls (sense probes). Table 1 also lists the relative abundance of AR expression in nuclei throughout the CNS in midshipman and highlights sites identified as part of the auditory and/or vocal system (also see Discussion).

Forebrain

Robust AR mRNA expression is found in discrete nuclei in the ventral telencephalon, including the dorsal, supracommissural, postcommissural, and intermediate nuclei of area ventralis (Vd, Vs, Vp, and Vi, respectively) (Figs. 6A–C, 7A). Similarly, prominent AR mRNA is seen in very specific regions of the dorsal telencephalon. Rostrally, the central zone of area dorsalis (Dc) is comprised of two distinct clusters of cells well-defined by AR mRNA expression: the slightly more medial group adjoins Dm while the ventrolateral group sits above Dp (Fig. 6C). In the caudal telencephalon, the posterior (Dm-p) and the central medial (Dm-cm) divisions of the medial zone of area dorsalis are sites of strong AR mRNA signal (Fig. 7A,B); and AR expression in Dc (Fig. 6C) is contiguous with Dm-cm.

Abundant AR mRNA is found throughout the preoptic area. Robust signal covers the extent of the anterior parvocellular preoptic area (PPa) (Fig. 6B,C) and the peripheral margin of the anterior magnocellular preoptic area (PM) (Fig. 6C), and is also over the more caudal gigantocellular division of PM (PMg) (Fig. 7A,B). Less intense expression is found in lateral and medial subdivisions of nucleus preglomerulosus (PGl/m) along the lateral aspect of the diencephalon (Fig. 7A,B). Medial nuclei of the anterior ventral hypothalamus contain abundant AR mRNA, including the ventral and anterior tuberal nuclei (vT, Fig. 7B; AT, Fig. 8A), and dorsal and ventral periventricular areas (Hd and Hv, Fig. 8A,B). The caudal extent of Hd and Hc, however, are devoid of AR mRNA expression (Fig. 9). Throughout its extent, the periventricular posterior tuberal nucleus (TPp, Fig. 8A,B) shows dense AR mRNA signal that appears contiguous with expression along the third ventricle and within the central and dorsal posterior nuclei of the thalamus (CP and DPo, Fig. 8B). Caudal to TPp, the posterior tuberal nucleus (TP) likely contains the greatest concentration of AR mRNA in the brain (Fig. 9); the ventricular midline just dorsal to TP contains lower but clear AR mRNA signal (arrows, Fig. 9A1).

Midbrain

In general, hybridization signal in the midbrain is lower and more diffuse compared to forebrain nuclei. Consistent AR mRNA expression is seen throughout the periventricular cell layer of the midbrain tectum (Pe, Figs. 8 - 10; see Brantley and Bass, 1988, for cytoarchitecture) and the periventricular cell layer of the torus semicircularis (arrows in TS, Fig. 10A1,A3,B1; see Bass et al., 2000, for cytoarchitecture). Hybridization signal is apparent at rostral levels along the dorsal tegmental midline (midline arrowhead, Fig. 10A1) and a region of the central gray previously identified as the periaqueductal gray (PAG) in midshipman (Fig. 10A) because of its shared neuroanatomical and neurophysiological traits with the same-named region in mammals (Goodson and Bass, 2002; Kittelberger et al., 2006). In the caudal midbrain, AR mRNA expression is consistently found over the band of cells that comprise the griseum centrale (GC) bordering the fourth ventricle (Fig. 10B).

Hindbrain and spinal cord

Similar to the midbrain, the rostral hindbrain shows diffuse AR mRNA signal along the fourth (IV) ventricle (arrows, Figs. 11, 12A). A distinct patch of AR expression is found over the cell plate of the medial octavolateralis nucleus (MED, Fig. 11), with additional AR signal at this level between the medial longitudinal fasciculus (MLF) and lateral lemniscus (ll) (arrow, Fig. 11A1). More caudally, at the level of the vagal lobe (VL), AR mRNA lines the fourth ventricle and continues ventrally into the vagal motor nucleus (Xm) and along the lateral edge of the MLF (Fig. 12A). Robust AR mRNA expression in the caudal hindbrain, comparable in density to that found in forebrain nuclei, is found over the vocal prepacemaker nucleus (VPP) within the ventrolateral reticular formation (Fig. 12A). Equally prominent AR expression is found overlying the VMN spanning the hindbrain-spinal cord boundary (Fig. 12B).

Inner ear

AR gene expression in the inner ear was initially demonstrated by RT-PCR (Fig. 4). The main end organ of hearing in midshipman and many other teleosts is the saccule (Cohen and Winn, 1967; Bass and McKibben, 2003). Using anatomical landmarks of the saccule (see Forlano et al., 2005; Fig. 13A), we determined that the expression for AR mRNA was concentrated over discrete patches of cells, likely support cells, just outside the hair cell layer (Fig. 13).

DISCUSSION

The current study demonstrates AR mRNA hybridization signal at all brain levels, providing anatomical support for androgen actions on vocal, auditory, and neuroendocrine circuits in

midshipman fish and the closely related toadfish, and in teleosts in general. Abundant AR mRNA expression was observed at forebrain, midbrain, and hindbrain-spinal cord levels of the descending vocal motor pathway as well as in sites of vocal-acoustic integration. Furthermore, our findings show the first evidence of AR mRNA in the inner ear of a vertebrate, in this case in the saccule, which is the main auditory division of the inner ear in many teleosts including midshipman fish (Bass and McKibben, 2003). Table 1 lists the relative abundance of AR expression in nuclei throughout the CNS in midshipman and highlights sites identified as part of the auditory and/or vocal system. Figure 1 summarizes AR mRNA expression in sites identified as part of the auditory and/or vocal system.

AR subtypes

Aside from the present study, the neuroanatomical distribution of AR mRNA expression in teleosts has been documented in the cichlid *Astatotilapia burtoni* (Harbott et al., 2007), and the zebrafish *Danio rerio* (Gorelick et al., 2008). In *A. burtoni*, the distribution of two AR subtypes, AR α and AR β , was documented mainly throughout the telencephalon and ventral hypothalamus. While both subtypes were highly expressed in the preoptic area, AR β was more widely distributed in the brain, notably in dorsal and central divisions of area dorsalis in the telencephalon, and found at higher expression levels in the forebrain and midbrain by quantitative (Q)RT-PCR. Although localization was not described in the hindbrain by ISH, transcripts of both subtypes were identified by QRT-PCR and expression of AR α was found to be significantly greater in the hindbrain and the pituitary gland. The sequence comprising the partial cDNA for midshipman AR shares highest identities with other teleost AR β s (Fig. 3), and its robust distribution in the dorsal telencephalon and widespread expression throughout the brain further supports that the midshipman AR described here is an AR β subtype. Also, the AR in the present study was first isolated from vocal muscle which is responsive to both testosterone and the nonaromatizable androgen 11-ketotestosterone (Brantley et al., 1993b; Lee and Bass, 2005), and therefore may contain one AR sensitive to different androgens, again similar to the broad binding affinity of AR β /AR2 described in other teleosts (Sperry and Thomas, 1999, 2000). Recent studies indicate a single AR gene in zebrafish that is also closest to AR β in sequence, has binding affinities to both testosterone and 11-ketotestosterone, and likely encodes a functional ortholog of the mammalian AR (Jorgensen et al., 2007; Gorelick et al., 2008; Hossain et al., 2008). Another cyprinid fish, *Spinibarbus denticulatus*, also expresses only one AR isoform; RT-PCR analysis showed AR in the olfactory bulb, telencephalon, and hypothalamus of males and females, but only in posterior brain regions (optic tectum, medulla, and spinal cord) in males (Liu et al., 2009).

The AR isoform most similar to AR β in fishes appears closest to that of other vertebrates in sequence homology and androgen binding and is the probable ancestral vertebrate AR (Sperry and Thomas, 1999; Harbott et al., 2007; Gorelick et al., 2008). A recent analysis proposes that basal teleosts such as *Anguilla* have retained both AR subtypes, while intermediate groups (e.g., salmonids and ostariophysians that includes zebrafish) secondarily lost one subtype and derived teleosts (percomorphs that include cichlids) have retained both subtypes (Douard et al., 2008). Given this pattern, midshipman would likely fall into the intermediate groups that express a single AR gene. However, there may be an 11-ketotestosterone-specific receptor yet to be identified in midshipman equivalent to AR β 2 identified in male stickleback kidney (Olsson et al., 2005), although this receptor may only be expressed in androgen-sensitive peripheral tissues.

Comparative neuroanatomical AR distribution with other teleosts

The widespread distribution of AR mRNA documented in the present study overlaps, in part, that described in cichlids and zebrafish (Harbott et al., 2007; Gorelick et al., 2008) and using steroid autoradiography in earlier studies of other teleosts, including toadfish, (Morrell et al.,

1975; Davis et al., 1977; Bass et al., 1986; Fine et al., 1996). Like *A. burtoni* (Harbott et al., 2007), midshipman show AR expression in Dc, but in addition have robust signal in the central medial (cm) and posterior (p) divisions of Dm. In contrast, zebrafish alone shows AR mRNA signal in the dorsolateral telencephalon (Gorelick et al., 2008). The expression in Dm-cm and Dm-p of midshipman borders a larger medial division of Dm that is retrogradely labeled following neurobiotin injection into the ventral tuberal region of the anterior hypothalamus (vT; Fig. 7A), a vocal site in midshipman that receives input from the ascending auditory system (Bass et al., 2000; Goodson and Bass, 2000a, 2002). A review of that original material (A. Bass, unpubl. obs.) shows retrogradely filled cells in Dm-cm and Dm-p, although very modest in comparison to the larger Dm region. Thus, AR mRNA expression may include vocal and/or auditory related regions of Dm.

Compared to studies in other teleosts, AR mRNA expression in supracommissural (Vs), postcommissural (Vp), and intermediate (Vi) nuclei of the ventral telencephalon has only been reported here in midshipman. Androgen-concentrating cells were also documented in the Vs of toadfish, but not other species (review in Fine et al., 1996). Fine et al. (1996) report androgen-concentrating cells in a restricted area of Dp that is most likely equivalent to Vi in the current study. Unlike *A. burtoni* (Harbott et al., 2007) and paradise fish (Davis et al., 1977), AR mRNA was not found in Vv in midshipman (this study) and toadfish (Fine et al., 1996). We also found robust AR expression in periventricular and posterior tuberal nuclei and along the periventricular optic tectum, like zebrafish (Gorelick et al., 2008) but unlike *A. burtoni* (Harbott et al., 2007). Consistent with all other teleosts so far studied (see above), midshipman have abundant AR expression in several divisions of the preoptic area and the ventral hypothalamus, including proposed homologs to mammalian arcuate (Hv; Cerda-Reverter et al., 2003; Forlano and Cone, 2007), para-ventricular (PM; Gilchrist et al., 2000; Kapsimali et al., 2001) (also see Forlano and Cone, 2007, and references therein), and ventromedial (AT; Goodson, 2005; Forlano et al., 2005; Forlano and Cone, 2007) hypothalamic nuclei. Similarly, AR mRNA is found highly expressed in amygdala homologs in the ventral telencephalon (Vi, Vp, Vs) (Northcutt and Braford, 1980; Northcutt, 1995), and a striatal homolog (Vd) (Wullimann and Mueller, 2004; Northcutt, 2006). Regions of Dm and Dc that show abundant AR mRNA may compare to either ventral or dorsal pallidum (see Braford, 1995; Wullimann and Mueller, 2004; Yamamoto and Ito, 2005; Northcutt, 2006; Yamamoto et al., 2007). This is consistent with findings across vertebrate groups, where AR expression is located in reproductive and limbic brain areas, including the preoptic area-anterior hypothalamus, as this conserved brain region is involved in sexual behavior and control of gonadotropin release (Goodson and Bass, 2001; De Vries and Simerly, 2002).

In the present study, additional sites of AR mRNA were reported for the first time in a teleost in the diencephalon (ventral and anterior tuber, central and dorsal posterior thalamus, lateral and medial divisions of nucleus preglomerulosus), midbrain (torus semicircularis, periaqueductal gray, griseum centrale), hindbrain (nucleus medialis, vagal motor nucleus, vocal prepacemaker and motor nuclei, reticular and periventricular areas) and in the saccule division of the inner ear. Androgen-concentrating cells labeled in the inferior reticular formation in toadfish (Fine et al., 1996) are probably equivalent to AR mRNA signal in midshipman vocal prepacemaker (A. Bass, unpubl. obs.).

Androgen-dependent plasticity in teleost vocal motor system

Testosterone and 11-ketotestosterone are elevated in the circulation during prespawning and spawning seasons in midshipman and toadfish (Modesto and Canario, 2003; Fine et al., 2004; Sisneros et al., 2004a), and influence the vocal motor system and behavior of midshipman and toadfish (see Introduction; Remage-Healey and Bass, 2006b; Forlano et al., 2007; Bass, 2008; Bass and Remage-Healey, 2008). AR expression in one or more vocal sites (Fig. 1A)

likely contributes to the known effects of androgens on the vocal system and vocal behavior (see Introduction). The mechanism by which androgens rapidly modulate the vocal pattern generator is unknown (Remage-Healey and Bass, 2004, 2006, 2007); however, these effects are blocked by cyproterone acetate (CA), a selective AR antagonist in fishes (Wells and Van Der Kraak, 2000). This implies that the mode of action involves a receptor or membrane binding site that contains a similar structure to a nuclear-like AR receptor (Remage-Healey and Bass, 2004). Interestingly, CA similarly blocks the rapid action of both testosterone and 11-ketotestosterone on vocal output, and may indicate that the same receptor is responsible for the effects of both androgens, although their sensitivities are tuned to a specific androgen in each morph/sex (Remage-Healey and Bass, 2007; Bass and Remage-Healey, 2008).

Comparisons with AR distribution in vocal tetrapods

Our results are consistent with evidence for expression of AR in vocal forebrain and brainstem nuclei, and vocal muscles across vertebrates (for review, see Bass and Remage-Healey, 2008). AR expression is well documented in vocal neurons in amphibians (Kelley, 1986; Perez et al., 1996), songbirds (Nastiuk and Clayton, 1995; Gahr and Wild, 1997; Gahr and Metzdorf, 1997; Bernard et al., 1999; Metzdorf et al., 1999; Gahr, 2001; Kim et al., 2004) non-songbirds (Shaw and Kennedy, 2002; Matsunaga and Okanoya, 2008), and reptiles (Tang et al., 2001). In primates, although extensive mapping of vocal pathways and associated motor nuclei is well documented (see Jurgens, 2002), AR expression associated with these areas has not been specifically investigated. However, extensive AR expression is found in vocal-related motor nuclei in rats (i.e., nucleus ambiguus, trigeminal motor, facial, hypoglossal) (Simerly et al., 1990) (see Jurgens, 2002, for vocal motor overview). Like midshipman fish (Goodson and Bass, 2002; Kittelberger et al., 2006), the midbrain PAG that activates hindbrain vocal nuclei in birds and mammals are abundant with ARs (see references above). Also like midshipman, amphibian preoptic and hypothalamic nuclei that project to brainstem vocal nuclei express high amounts of AR (Kelley, 1986). Lastly, like vocal motor and pacemaker nuclei in midshipman, the vocal motor and premotor nuclei in songbirds express AR (see Gahr and Wild, 1997). Thus, in addition to brainstem-spinal vocal motor circuitry itself (Bass et al., 2008), AR expression within vocal nuclei also appears to be an ancestral, conserved phenotype of vertebrates (also see Bass and Remage-Healey, 2008).

Steroid-dependent plasticity in the auditory system

Improved temporal encoding of the frequency components of male calls have been recorded from eighth nerve, saccular afferents in female midshipman fish during reproductive periods, paralleling seasonal increases in circulating testosterone and 17 β -estradiol (Sisneros and Bass, 2003; Sisneros et al., 2004a). Both testosterone and 17 β -estradiol treatments induce a reproductive-like auditory phenotype in nonreproductive females, and the testosterone effect could be due to its local conversion to 17 β -estradiol by aromatase (Sisneros et al., 2004b). In support of this hypothesis, estrogen receptor (ER) α mRNA was identified just outside the hair cell layer and aromatase-immunoreactive ganglion cells were found in the branch of the eighth nerve proximal to the saccule's sensory epithelium (Forlano et al., 2005). Similar to ER α , we show AR mRNA expressed in cells just outside the hair cell layer of the sensory epithelium, in support of androgen-dependent plasticity in the inner ear. While these AR-positive cells, likely support cells, could indirectly affect the properties of the hair cells, androgens may also act directly on hair cells; mRNA levels may either be expressed below the threshold of detection of ISH or fluctuate seasonally and therefore have been potentially missed in our sampling. It is likely, however, that androgens function, in part, to upregulate local aromatase in eighth nerve ganglion cells, as found in the brain (Forlano and Bass, 2005b), thereby increasing local 17 β -estradiol production proximal to ERs (also see Forlano et al., 2005).

Several nuclei in the central auditory system (Bass et al., 2000) consistently expressed AR mRNA (Table 1; Fig. 1B), including the torus semicircularis (TS) that is the homolog of the tetrapod inferior colliculus (Bass et al., 2005), and several sites in the diencephalon (CP, AT, PGI) that receive direct input from the auditory torus (Bass et al., 2000, 2001). AR mRNA is also highly expressed in preoptic-anterior hypothalamic (PPp, PPa, vT) and ventral telencephalic (Vd, Vp, Vs) nuclei that receive input from the auditory thalamus (CP) (Goodson and Bass, 2002). AR mRNA in the lateral line recipient hindbrain nucleus medialis suggests the potential influence of androgens on the auditory-like vocal encoding abilities of the lateral line system (Weeg and Bass, 2000, 2002). With the exception of the auditory-recipient telencephalic nuclei, AR mRNA is expressed more robustly than ER α (Forlano et al., 2005) throughout central auditory circuitry, which suggests that androgens have an important role for modulation of auditory function in midshipman (also see next section).

AR mRNA has been documented in the central auditory system of a vocal lizard, *Gecko gecko* (Tang et al., 2001) and in rats (Simerly et al., 1990), while the TS of the frog *Xenopus* concentrates androgens (Kelley, 1980), but in all cases the functional significance has not been investigated. Interestingly, humans with Kennedy's disease (also known as X-linked bulbospinal muscular atrophy) that is caused by a mutation on the AR gene (Palazzolo et al., 2008) show impaired hearing accompanying abnormal brainstem auditory evoked potentials (Lai et al., 2007).

AR in relation to aromatase and estrogen receptor alpha expression

Compared to mammals, teleosts have exceptionally high aromatase and AR levels in brain tissue (Pasmanik and Callard, 1988; Callard et al., 1990). Testosterone has a high affinity for aromatase and therefore high levels of brain aromatase may significantly reduce brain androgen levels that may, in turn, necessitate high AR levels to activate AR signaling pathways (Pasmanik and Callard, 1988).

Table 1 compares the relative abundance of AR mRNA (this report) with aromatase mRNA/protein (Forlano et al., 2001) and ER α mRNA (Forlano et al., 2005) in midshipman fish, although we fully recognize the need for double-labeling experiments to demonstrate cellular colocalization. In general, AR and aromatase are abundantly coexpressed in forebrain nuclei in the telencephalon, preoptic area, hypothalamus, and thalamus (aromatase-positive glial cells along ventricles are adjacent to and have processes extending into CP, DPo and Tpp; see fig. 2 in Forlano et al., 2001, and fig. 12 in Forlano et al., 2005). Similar to songbirds (Bernard et al., 1999; Metzendorf et al., 1999), AR expression is comparatively more widespread and found in greater abundance than ER in vocal nuclei (indicated in Table 1). ARs are also more prominent in the central auditory system (Table 1). Thus, there appears to be a greater relative abundance of "classical" androgen to estrogen receptors throughout vocal-acoustic nuclei, although the distribution of other ER subtypes have yet to be defined in midshipman.

Our previous neuroanatomical studies show that testosterone upregulates brain aromatase expression, particularly in the VMN (Forlano and Bass, 2005b; Bass and Forlano, 2008). This is consistent with abundant AR expression along the dorsal rim of the VMN (Fig. 12B) where aromatase-positive glial cell bodies are similarly abundant (see Forlano et al., 2001, for aromatase), suggesting colocalization of AR and aromatase. ARs have been identified in glial cells in mammals (for review, see DonCarlos et al., 2006; Sarkey et al., 2008), although it is not known whether these same cells also express aromatase. The results are also consistent with the hypothesis that local estrogen production in the VMN, dependent on aromatization of testosterone by glial cells, contributes to estrogen modulation of the vocal hindbrain-spinal cord (Remage-Healey and Bass, 2004, 2007). The robust expression of AR over VMN glial cells contrasts sharply with the more rostral VPP that shows AR expression over neuronal somata themselves and not in an aromatase-glial-like pattern (P. Forlano and A. Bass, unpubl.

obs.). Given VPP's important role in vocal patterning (Chagnaud et al., 2009), the VPP is a likely a specific site underlying androgen action in the vocal system (Remage-Healey et al., 2004, 2007).

Androgen receptor expression in relation to neuropeptides

While androgens and steroid hormones in general increase vocal motor output (Remage-Healey and Bass, 2004), the neuropeptides arginine vasotocin (AVT) and isotocin (IT) (homologs to mammalian vasopressin and oxytocin, respectively) inhibit evoked fictive vocalizations in midshipman (Goodson and Bass, 2000a). Due to the opposing actions of androgens and AVT/IT on vocal motor output, androgens may function, in part, to inhibit AVT/IT release (Bass and Remage-Healey, 2008). Indeed, AR is highly expressed in all vocal-related nuclei that contain AVT/IT-ir neurons (POA, Fig. 1A) and in hypothalamic vocal stimulation sites that are responsive to AVT application and contain AVT/IT-ir terminals (AT, vT; Fig. 1A) (Goodson and Bass, 2000a,b; Goodson et al., 2003).

CONCLUSIONS

It is only through the recent delineation of the functional signature of discrete neuronal populations, for example, GnRH preoptic neurons regulating gonadal size (see Harbott et al., 2007) and vocal-auditory circuits (see Introduction), that AR localization in the CNS of teleosts becomes especially salient in the context of identifying the mechanisms underlying steroid-dependent behaviors. In addition to localizing abundant AR mRNA in preoptic-hypothalamic regions (POA-AH) with ubiquitous involvement in vertebrate reproduction (Goodson and Bass, 2001; Remage-Healey and Bass, 2006b; Forlano et al., 2007; Bass and Forlano, 2008), the current study also demonstrates AR mRNA localization in vocal and auditory circuits that include the POA-AH (Goodson and Bass, 2002). Studies of vocal fish provide the opportunity to show how androgens influence vocal-acoustic networks that are conserved between fish and tetrapods (Bass et al., 2005, 2008) and include sites of AR mRNA expression (this report). Thus, seasonal breeding fish and tetrapods alike that rely on vocal-acoustic communication show an intricate link between reproductive steroid cycling, audition, and vocalization via steroid, including androgen, receptors acting in the central and peripheral nervous system.

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Abbreviations

ac	Anterior commissure
AT	Anterior tuberal nucleus
C	Cerebellum
CA	Cerebral aqueduct
Cc	Cerebellar crest
Cg	Granular layer of the corpus of the cerebellum
Cm	Molecular layer of the corpus of the cerebellum
CP	Central posterior nucleus of the thalamus
D	Area dorsalis of the telencephalon
Dc	Central zone of D

Df	Diffuse nucleus of the hypothalamus
DI	Lateral zone of D
Dm	Medial zone of D
Dm-cm	Central medial division of Dm
Dm-p	Posterior division of Dm
DO	Descending octaval nucleus
Dp	Posterior zone of D
DPo	Dorsal posterior nucleus of the thalamus
Eg	Eminentia granularis
F	Forebrain
G	Nucleus glomerulosus
GC	Griseum centrale
H	Hindbrain
Ha	Habenula
HC	Hair cell layer
Hd	Dorsal periventricular hypothalamus
HoCo	Horizontal commissure
Hv	Ventral periventricular hypothalamus
III	Third ventricle
IL	Inferior lobe of the hypothalamus
Isth/Teg	Isthmal and midbrain tegmental region
IV	Fourth ventricle
LH	Lateral hypothalamus
II	Lateral lemniscus
M	Midbrain
MED	Cell plate of medial octavolateralis nucleus
MLF	Medial longitudinal fasciculus
nMLF	Nucleus of the medial longitudinal fasciculus
OB	Olfactory bulb
OE	Octavolateral efferent nucleus
oc	Occipital nerves
ot	Optic tract
P	Medial pretoral nucleus of the pretectum
PAG	Periaqueductal gray
Pe	Periventricular cell layer of the midbrain tectum
PGl	Lateral division of nucleus preglomerulosus

PGm	Medial division of nucleus preglomerulosus
Pit	Pituitary
PM	Magnocellular preoptic nucleus
PMg	Gigantocellular division of PM
PPa	Anterior parvocellular preoptic nucleus
PPp	Posterior parvocellular preoptic nucleus
PoC	Posterior commissure
RF	Reticular formation
SE	Sensory cell epithelium of the saccule
SO	Secondary octaval nucleus
SV	Saccus vasculosus
T	Telencephalon
TEG	Tegmentum
TeM	Midbrain tectum
TL	Torus longitudinalis
TP	Posterior tuberal nucleus
TPp	Periventricular posterior tuberal nucleus
TS	Torus semicircularis
v	Ventricle
V	Area ventralis of the telencephalon
Vc	Central nucleus of V
Vd	Dorsal nucleus of V
Vg	Granular layer of the valvula
Vi	Intermediate nucleus of V
VI	Ventrolateral nucleus of the thalamus
VL	Vagal lobe
Vm	Molecular layer of the valvula
VM	Nucleus ventromedialis
VMN	Vocal motor nucleus
Vp	Postcommissural nucleus of V
VPG	Vocal pattern generator
VPN	Vocal pacemaker neurons
VPP	Vocal prepacemaker nucleus
Vs	Supracommissural nucleus of V
vT	Ventral tuberal hypothalamus
Vv	Ventral nucleus of V

VIII Eighth nerve

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Figure 1.

Side views of the brain showing vocal motor (A) and central auditory (B) systems in batrachoidid fish (midshipman and toadfish) (modified from Bass and McKibben, 2003; Kittelberger et al., 2006). Solid dots represent somata, and lines represent axonal projection pathways. Two connected dots indicate reciprocal connections. Shaded areas indicate localization of AR expression. A: Descending vocal motor pathways (see Bass and Baker, 1990; Bass et al., 1994; Goodson and Bass, 2002; Kittelberger et al., 2006). Preoptic (POA) and ventral (vT) and anterior (AT) tuberal nuclei in the hypothalamic forebrain project to the periaqueductal gray (PAG) in the midbrain which then connects to the vocal pattern generator (VPG) in the hindbrain-spinal cord. The VPG consists of vocal prepacemaker (VPP), pacemaker (VPN), and motor (VMN) nuclei. The VMN projects directly via occipital nerve roots to sound-producing muscles on the swim bladder (shown in ventral, dissected view). The arrow points to a vocalization produced by the swim bladder, a typical “grunt train” often produced during nest defense by a type I male (Brantley and Bass, 1994). B: Central auditory system (see Bass et al., 2000, 2001). Natural sounds, represented by a “grunt train,” are detected by the inner ear which projects via the VIIIth nerve to octaval nuclei in the hindbrain and further to the auditory midbrain torus semicircularis (TS). Shown are nuclei interconnected with TS. The dorsal thalamic central posterior nucleus (CP) contains reciprocal connections to the dorsomedial (Dm) and ventral supracommissural (Vs) telencephalon which also receives input from the anterior hypothalamus-posterior tubercle (for nomenclature, see Braford and Northcutt, 1983). TS and CP also connect to vocal motor nuclei in the forebrain (anterior hypothalamus and POA) and midbrain (PAG and isthmal/tegmentum), while auditory-recipient octaval nuclei in the hindbrain connect to the VPG (also see Bass et al., 1994; Goodson and Bass, 2002).

GAGGCGTCAGGGTGTCTTATGGCGCGCTCACCTGCGGCAGTTGCAAGGTG
E A S G C H Y G A L T C G S C K V
 TTCTTCAAGAGAGCTGCAGAAGGCAAACAGAAATACCTGTGTGCAAGCAAA
 F F K R A A E G K Q K Y L C A S K
 AACGACTGCACAATCGATAAGCTAAGAAGAAAGAACTGCCCTTCCTGTCTGT
 N D C T I D K L R R K N C P S C R
 CTAAAGAAATGTTTTGAAGCTGGAATGACCCTTGAGCACGGAAACTGAAG
 L K K C F E A G M T L G A R K L K
 AAGATTGGCCAACAGAAGAACCCTGATGAGGACTTTCCTCTCCAGGAGTCT
 K I G Q Q K N P D E D F P L Q E S
 GTTGAGATCATCCAGAACATGTCTCTAAAGGTGGTCTGAACTTCAACTCC
 V E I I Q N M S P K G G L N F N S
 CACGTGGTCTTCCTCAACATCTTGAATCCATAGAGCCCGAGGTGGTGAAC
 H V V F L N I L E S I E P E V V N
 GCCGGACACGACTGTTGCCAGCCAGACTCTGCGGCCACTCTGCTCACCAGC
 A G H D C C Q P D S A A T L L T S
 CTCAATGAGCTCGGAGAGCGACAGCTGGTCAAGGTGGTCAAGTGGGCGAAG
 L N E L G E R Q L V K V V K W A K
 GGG
G

Figure 2.

Nucleotide and deduced amino acid sequence of AR partial cDNA clone isolated from midshipman brain. Underlined amino acids and basepairs correspond to sequence derived from degenerate primers.

Figure 3.

Alignment of deduced amino acid sequences of midshipman with other representative vertebrate ARs: teleost (tilapia, wrasse, stickleback, croaker, trout, zebrafish, and eel), amphibian (*Xenopus*), mammalian (human and mouse) and avian (zebra finch). Black and gray shading indicate identical and similar amino acids to midshipman AR, respectively. The first and last eight amino acids are conserved regions to which degenerate primers were made for RT-PCR. The entire sequence falls within the DNA (*) and ligand binding domain (**) and is up to 90% conserved with other teleosts.

Figure 4.

Using midshipman-specific primers, a predicted 150-bp product containing AR transcripts was identified by RT-PCR in a forebrain region (F), a middle CNS region including midbrain and caudal diencephalon (M), the cerebellum (C), hindbrain and rostral spinal cord (H), vocal muscle (VM), and saccular epithelium of the inner ear (E) of individual adult midshipman. Shown is a representative type I male.

Figure 5.

Dorsal view of exposed brain and inner ear of a midshipman indicating levels (rostral-caudal) of transverse sections for the brain atlas of androgen receptor distribution shown in Figures 6-12. C, cerebellum; M, midbrain; OB, olfactory bulb; OC, occipital nerve roots; T, telencephalon; SE, saccular epithelium of the inner ear; VIII, eighth nerve. Modified from Forlano et al. (2005). Scale bar = 1.5 mm.

Figure 6.

AR mRNA expression in the ventral telencephalon and rostral preoptic area. In this figure and in Figures 6-12, neuroanatomical localization of in situ hybridization signal is shown for the same section in low-magnification darkfield (left) and brightfield Nissl-stained sections (middle) and higher-magnification brightfield (right). **A1–3:** Robust AR expression in dorsal nucleus of area ventralis (Vd). **B1–B3:** High AR mRNA expression in the supracommissural nucleus of area ventralis (Vs) and anterior parvocellular preoptic area (PPa), part of the forebrain vocal-acoustic complex (see Fig. 1A,B; Table 1). **C1–C4:** Strong AR expression in the dorsal pallium in the central zone of area dorsalis (Dc) as well as throughout the extent of the PPa and the postcommissural nucleus of area ventralis (Vp). Scale bar = 500 μ m for panels 1, 2 in A–C; 100 μ m, 200 μ m, and 100 μ m panels in A3, B3, and C3–C4, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 7.

AR mRNA expression in the dorsal telencephalon and caudal preoptic area. **A1–A5:** The posterior (p) and central medial (cm) divisions of the medial zone of area dorsalis (Dm-p and Dm-cm), the intermediate nucleus of area ventralis (Vi), and the gigantocellular division of the magnocellular preoptic area (PMg) show strong AR mRNA expression. **B1–B4:** In addition to strong hybridization signal in the dorsal pallium (Dm-p, Dm-cm) and ventricular zone adjacent to PMg, the ventral tuberal hypothalamus (vT), part of the descending vocal motor pathway (see Fig. 1), shows robust AR expression. Scale bar = 500 μ m for panels 1, 2 in A,B; 100 μ m for A3–A5 and B3–B4. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 8.

AR mRNA expression in the diencephalon and rostral hypothalamus. **A1–A3:** Dorsal (Hd) and ventrolateral periventricular (Hv) nuclei of the hypothalamus show abundant AR; lower levels are found in the anterior tuberal nucleus (AT), part of the forebrain vocal-acoustic complex (see Fig. 1). **B1–B4:** Diffuse AR mRNA expression is found in the auditory central posterior nucleus (CP, see Fig. 1), the dorsal posterior (DPo) nucleus of the thalamus, and the periventricular layer of the midbrain tectum (Pe), as well as along the third ventricle (III) including the periventricular posterior tuberal nucleus (TPp, also see A). Scale bar = 500 μ m for panels 1, 2 in A,B; 200 μ m and 100 μ m for A3 and B3–B4, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 9.

A1–A4: The posterior tuberal nucleus (TP), shown in high magnification at two levels, has perhaps the highest abundance of AR mRNA in the central nervous system. Also note consistent signal (arrows) along the ventricular midline in the thalamus (midline) and in the periventricular cell layer of the tectum (Pe). Scale bar = 500 μm for panels A1–A2; 100 μm for A3–A4. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 10.

AR mRNA expression at two midbrain levels. **A1–A4:** Hybridization signal in the periaqueductal gray (PAG), part of the descending vocal motor pathway (see Fig. 1), and the periventricular torus semicircularis (TS, arrows), tectum (Pe) and dorsal tegmental midline (arrowhead). **B1–B3:** AR mRNA expression in a more caudal section of the midbrain which also demonstrates signal in periventricular TS (arrows), tectum's Pe, and griseum centrale (GC). Scale bar = 500 μ m for panels 1, 2 in A,B; 100 μ m and 200 μ m for A3–A4 and B3, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 11.

A1–A4: AR mRNA expression in the lateral line recipient nucleus medialis (MED). Also notice diffuse signal along the fourth ventricle (IV) and lateral to the medial longitudinal fasciculus (MLF) (arrows). Scale bar = 500 μ m for A1–A2; 200 μ m and 100 μ m for A3 and A4, respectively. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 12.

AR mRNA expression in the vocal, hindbrain-spinal cord pattern generator. **A1–A4:** The vocal prepacemaker nucleus (VPP) shows robust AR expression; also consistent but diffuse mRNA signal found along the ventricle (IV). **B1–B3:** Strong AR mRNA expression forms a cap around the dorsal periphery of the vocal motor nucleus (VMN). Scale bar = 500 μm for panels 1, 2 in A,B; 100 μm in A3; 200 μm in A4,B3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 13.

AR mRNA expression in the inner ear of female midshipman. **A:** The hair cell layer (HC) of the sensory epithelium of the saccule is delineated by a hair cell-specific antibody (brown), overlaying Nissl stained support cells (see Forlano et al., 2005, for methods). **B:** Arrowhead indicates AR mRNA (clusters of silver grains) over cells just outside the HC layer. **C1–C3:** Examples of hybridization specificity in the inner ear in a similar location as B. **C2–C3:** Higher magnification of cells indicated by arrowheads in C1. Scale bar = 100 μ m in A,B; 50 μ m in C1; 30 μ m in C2–C3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Comparative Distribution of Androgen Receptor (AR), Aromatase (Estrogen Synthase), and Estrogen Receptor alpha (ER α) in the Brain of the Plainfin Midshipman, *Porichthys notatus*

TABLE 1

Brain area	AR mRNA	Aromatase mRNA/ protein / $\uparrow$		ER α mRNA ²
		AR mRNA	Aromatase mRNA/ protein / $\uparrow$	
Ventral telencephalon				
Olfactory bulb (OB)	-		+++	+
Ventral nucleus (Vv) AUDITORY	-		+++	+
Supra commissural nucleus (Vs) AUDITORY, VOCAL	++		+++	+
Postcommissural nucleus (Vp) AUDITORY	++		+++	+
Dorsal nucleus (Vd)	+++		+++	-
Intermediate nucleus (Vi)	+++		+++	-
Dorsal telencephalon				
Central zone (Dc)	++		++	+
Central medial division of medial zone (Dm-cm)	++		++	++
Posterior division of medial zone (Dm-p) AUDITORY, VOCAL	+		++	+
Dorsal posterior zone (Dp)	-		++	+
Preoptic area				
Anterior parvocellular (PPa) AUDITORY, VOCAL	++		+++	++
Posterior parvocellular (PPp) AUDITORY, VOCAL	++		+++	-
Magnocellular (PM/PMg)	++		+++	++
Ventral hypothalamus				
Anterior tuberal (AT) AUDITORY, VOCAL	++		+++	++
Ventral tuberal (vT) AUDITORY, VOCAL	++		+++	-
Periventricular (Hv/Hd)	++		+++	++
Thalamus				
Central posterior nucleus (CP) AUDITORY	+ ^{*$\uparrow$}		+++	+
Dorsal posterior (DPo)	+ ^{*$\uparrow$}		+++	+
Nucleus preglomerulosus (PGL/m) AUDITORY	+		+	-
Periventricular nucleus of posterior tuberal (TPp)	++ ^{*$\uparrow$}		+++	+
Posterior tuberal nucleus (TP)	+++		+++	-
Pineal	-		++	++

Brain area	Aromatase mRNA/ protein ^{†‡}		
	AR mRNA	ERα mRNA ²	
Brainstem			
Periaqueductal gray (PAG) VOCAL	+	+++	-
Mesencephalic tectum (TeM)	+	-	+
Medial longitudinal fasciculus (MLF) (peri)	* ^{†‡} +	++	-
Nucleus of medial longitudinal fasciculus (nMLF)	+	+++	+
Periventricular cell layer of TS (Pe) AUDITORY	++	-	-
Griseum central (GC)	* ^{†‡} +	+++	-
Medial octavolateralis nucleus (MED) LATERAL LINE	+	-	-
Vagal motor nucleus (Xm)	* ^{†‡} +	+	-
Vocal prepacemaker (VPP) VOCAL	++	-	-
Vocal motor nucleus (VMN) VOCAL	* ^{†‡} ++	+++	+
Peripheral tissues			
Saccular epithelium (inner ear) AUDITORY	+	++	+
Vocal muscle VOCAL	++	-	-

¹ Forlano et al. (2001)

² Forlano et al. (2005). See Discussion for auditory, vocal, and lateral line references.

^{†‡} Glial expression

*^{†‡} similar pattern to glial aromatase expression; +++, ++, + = relative levels of hybridization signal.