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Distribution of Estrogen Receptor Alpha mRNA in the Brain and Inner Ear of a Vocal Fish with Comparisons to Sites of Aromatase Expression

PAUL M. FORLANO, DAVID L. DEITCHER, AND ANDREW H. BASS*
Department of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853

ABSTRACT

Among vertebrates, teleost fish have the greatest capacity for estrogen production in the brain. Previously, we characterized the distribution of the estrogen-synthesizing enzyme aromatase in the brain of the midshipman fish. Here, we investigated the distribution of estrogen receptor alpha (ERα). A partial cDNA of ERα was cloned and used to generate midshipman-specific primers for RT and real-time PCR which identified transcripts in liver and ovary, the CNS, and the sensory epithelium of the main auditory endorgan (sacculus). In situ hybridization revealed abundant expression throughout the preoptic area, a vocal-acoustic site in the hypothalamus, amygdala homologs of the dorsal pallium, the pineal organ, the inner ear, the pituitary, and the ovary. Weaker expression was found in the midbrain's nucleus of the medial longitudinal fasciculus and in the dimorphic vocal motor nucleus. ERα expression in the pineal, gonad, and pituitary axis may function to time seasonal abiotic cues to reproductive state, while expression in the vocal motor and auditory systems support neurophysiological evidence for estrogen as a modulator of vocal motor and auditory encoding mechanisms in midshipman fish. While ERα is restricted to specific nuclei, aromatase expression is abundant in glial cells throughout the entire forebrain, and high in midbrain and hindbrain – spinal vocal regions. The only site of aromatase-containing neurons is in the peripheral auditory system, where it is localized to ganglion cells in the auditory nerve. Estrogen production proximal to ERα-positive neurons may provide for focal sites of estrogen effects on reproductive-, vocal-, and auditory-related neurons. J. Comp. Neurol. 483: 91–113, 2005.

Indexing terms: teleost; estradiol; preoptic area; pineal; vocal-motor; auditory

Among vertebrates, teleost fish have the highest brain levels of the estrogen-synthesizing enzyme aromatase (Callard et al., 1990). Previously, we characterized the anatomical localization, steroid regulation, and seasonal expression of aromatase in the brain of the midshipman fish *Porichthys notatus* (Forlano et al., 2001; Forlano and Bass, 2003), a well-studied species for the neural and endocrinological mechanisms of vocal-acoustic communication (Bass and McKibben, 2003). Aromatase is localized to glial cells in all brain areas and is most abundant in the forebrain preoptic and ventricular areas as well as in hypothalamic nuclei and along the third ventricle in the diencephalon (Forlano et al., 2001). Aromatase expression in the brainstem is localized along the periaqueductal gray in the midbrain and in the hindbrain vocal motor region, where its expression pattern is sexually dimorphic (Schlinger et al., 1999; Forlano et al., 2001; Bass and Forlano, 2005). In the forebrain of other vertebrate groups, highest levels of aromatase are consistently expressed in preoptic areas of the hypothalamus and limbic brain structures, i.e., areas involved in reproductive and sexual behaviors (for reviews, see Balthazart and Ball,

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*Correspondence to: Andrew H. Bass, Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14853. E-mail: ahb3@cornell.edu

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1998; Lephart et al., 2001). In contrast to teleosts, aromatase expression in tetrapods appears exclusive to neurons except under brain-injury conditions (Garcia-Segura et al., 2003).

The initial goal of the current study was to localize brain areas that express estrogen receptors as a first step to identify potential sites of interactions with estradiol produced from brain aromatase. We focused on estrogen receptor alpha expression (ERα mRNA) because it is the best-characterized of all ER forms (Toran-Allerand, 2004), its sequence and, in some cases, its brain expression pattern is known from a broad range of teleosts (Andreassen et al., 2003; Choi and Habibi, 2003; Hawkins et al., 2000; Huang et al., 2002; Kawahara et al., 2000; Kim et al., 2002; Menuet et al., 2001, 2002; Sabo-Attwood and Denslow, 2003; Teves et al., 2003), and ERα, but not ERβ, is upregulated by circulating estradiol levels (Sabo-Attwood et al., 2004).

During the early stages of this work, neurophysiological studies provided added functional context for the documentation of ERα localization in both the central and peripheral nervous systems. First, other studies of midshipman fish showed that 17β-estradiol can rapidly modulate vocal motor patterning in the hindbrain-spinal region that includes the expansive vocal pacemaker-motor neuron circuit in midshipman fish (Remage-Healey and Bass, 2004). Thus, we investigated whether ERα is expressed in this same brain region that also shows abundant aromatase expression. Second, we have also since found for midshipman fish that 17β-estradiol (and testosterone) can induce changes over the course of about one month in the frequency encoding properties of the eighth nerve afferents that innervate the sacculus, the main auditory endorgan of the inner ear in this group of teleosts (Sisneros et al., 2004b). Thus, we also tested the hypothesis whether these effects correspond to ERα localized to either the auditory periphery or central auditory nuclei.

We now report ERα mRNA in novel areas in the teleost brain, including amygdala homologs in the dorsal pallium, the pineal stalk, vocal and auditory nuclei in the forebrain and brainstem, and in the peripheral auditory system. As in other teleosts and vertebrates in general, abundant ERα mRNA expression also occurs in preoptic and hypothalamic areas. Together, these findings support estradiol as an important steroid that can modulate neurons directly involved in intraspecific communication during reproductive behaviors in the plainfin midshipman fish.

MATERIALS AND METHODS

Animals

Midshipman fish were collected from field sites in Tomales Bay, CA, during the spawning season (May–August 2002) or in Monterey Bay off Moss Landing, CA, at other times of the year (2002) and maintained in seawater tanks until sacrificed shortly afterwards (see Sisneros et al., 2004a, for a description of the reproductive and nonreproductive time periods). Given the known dependence of ERα expression on circulating estradiol levels (see Discussion), the animals chosen for study were from the spring and summer months when plasma levels of estradiol, and 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 ERα

Methods were previously reported for cloning of a partial cDNA for midshipman ERα (Sisneros et al., 2004b)

Abbreviations

ac	anterior commissure
aPit	anterior pituitary
AT	anterior tuberal nucleus
C	cerebellum
CA	cerebral aqueduct
CP	central posterior nucleus of the thalamus
D	area dorsalis of the telencephalon
DC	central zone of D
DF	diffuse nucleus of the hypothalamus
DL	dorsolateral zone of D
DM	medial zone of D
DMcm	central medial division of DM
DM	medial zone of D
DMP	posterior division of DM
DP	posterior zone of D
DPo	dorsal posterior nucleus of the thalamus
F	forebrain
GC	ganglion cell
H	hindbrain
HA	habenula
HC	hair cell layer
Hd	dorsal periventricular hypothalamus
HoCo	horizontal commissure
Hv	ventrolateral nucleus of the hypothalamus
LH	lateral hypothalamus
M	midbrain
MLF	medial longitudinal fasciculus
nMLF	nucleus of the medial longitudinal fasciculus
OB	olfactory bulb
oc	occipital nerves
ot	optic tract
PPa	anterior parvocellular preoptic nucleus
PGl	lateral division of nucleus preglomerulosus
PGm	medial division of nucleus preglomerulosus
PM	magnocellular preoptic nucleus
PoC	posterior commissure
pPit	posterior pituitary
PPp	posterior parvocellular preoptic nucleus
PVO	paraventricular organ
PS	pineal stalk
RF	reticular formation
SE	saccular epithelium
SMN	sonic motor nucleus
SV	saccus vasculosus
T	telencephalon
TD	torodiencephalic bundle
TeO	optic tectum
TL	torus longitudinalis
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
VL	ventrolateral nucleus of the thalamus
VP	postcommissural nucleus of V
Vs	supracommissural nucleus of V
VT	ventral tuberal hypothalamus
Vv	ventral nucleus of V
VIII	eighth nerve

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: GTNGARGGNATGGCNGARATHTTYGAYATG; reverse: RTCRTANAGNGGNACYTTRTTYTTRCAYTT) were designed based on a highly conserved region in the steroid binding domain of the ERα sequence of six known teleost species and a partial cDNA clone (363 bp) of ERα was amplified from liver tissue of a female midshipman undergoing gonadal recrudescence (GenBank Accession no. AY466470).

RT-PCR and relative quantitative real-time PCR

Internal, midshipman-specific primers (forward: CTGCTGGCTACCACCGCCCGCTTCCGCATG; reverse: CATGCTGTACAGGTGTTCCATGCCTTTGTT) were employed to identify ERα mRNA transcripts (303 bp) from reverse-transcribed RNA of ovary and liver (positive controls), pooled forebrain, midbrain, and hindbrain from six females undergoing gonadal recrudescence (during the natural peak of plasma circulating levels of 17β-estradiol and testosterone; see Sisneros et al., 2004a; also see Discussion), and pooled saccular epithelial tissue from four females ovariectomized and implanted with 17β-estradiol and allowed to survive 4–6 weeks (see Sisneros et al., 2004b). Total RNA was isolated from various tissues and DNase treated (Ambion, Austin, TX) according to the manufacturer's instructions, after which 2.2 μg of RNA was reverse-transcribed as previously reported. PCR was performed in a final volume of 50 μl containing 1× reaction buffer, 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM each primer, 0.5 μl Tfl DNA polymerase, and 2 μl of first-strand cDNA reaction. The reaction was run under the following conditions: 94°C for 5 minutes followed by 25 or 35 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute, and a final extension step at 72°C for 10 minutes. Negative controls included RNA from the above samples that had not been reverse-transcribed as template for the above PCR reaction, as well as a no-template control. Reactions were run on a 2% agarose gel stained with ethidium bromide to detect presence of transcripts in the various tissues.

Relative quantitation of ERα expression was based on the model by Pfaffl (2001), which is based on the relative expression of a target gene versus a reference gene. We used highly conserved sequences for primers, forward: TTGAGACCTTCAACACCCC, and reverse: ACTCCTGCTTGCTGATCCAC (100% match to all vertebrate species in GenBank) to amplify a 648 bp sequence of β-actin from midshipman liver cDNA for the reference gene. Thermocycler settings were as above except the annealing temperature was 50°C for 35 cycles. Three (50 μl) reactions were pooled and purified with a Concert Kit (Life Technologies, Gaithersburg, MD) per the manufacturer's instructions and directly sequenced at the Cornell University Biotechnology Sequencing facility.

For relative quantitative real-time PCR (liver, ovary, saccular epithelium, forebrain, midbrain, and hindbrain), forward (TATTCGATATGCTGCTGGCT) and reverse (AGGCTCCGGAGTTGAGTAAG) ERα primers or forward (CCCATCTATGAGGGCTATGC) and reverse (ATCGCGCACAATTTCTCTCT) β-actin primers were used at a concentration of 0.1 μM and added to qPCR Mastermix Plus for SYBR Green I reagents (Eurogentec, Philadelphia, PA) with 2 μl of first-strand cDNA reaction (as above). Samples were run in triplicate along with no template controls. Thermocycler settings were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, 52°C for 30 seconds, and 72°C for 30 seconds. Lastly, a denaturation step of 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds was used to generate a dissociation plot to confirm the presence of a single product in each reaction tube. Data were analyzed with ABI software (SDS 2.0; Applied Biosystems, Foster City, CA).

In situ hybridization (ISH)

The procedure for in situ hybridization detection of ERα mRNA was similar to our previous study for aromatase mRNA (Forlano et al., 2001). 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 0.1 M phosphate buffer (PB; pH 7.2). Brains (four females and two males), ovary (three females), and saccular epithelium of the inner ear (five 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 μm in the transverse plane for brain tissue and at 20 μm in the oblique plane for the inner ear; alternate sections were collected onto Superfrost Plus slides (Erie Scientific, Portsmouth, NH) and stored in −80°C until processed. Prior to hybridization, slides were equilibrated to room temperature for 10 minutes, washed 2 × in 0.1 M potassium phosphate-buffered saline (KPBS; pH 7.2), and placed into freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine 2 × 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 124–164 (CGTCGTGCAGCGGCTCCATTGTGCTGGTGCAGAAGGAAAA), 174–214 (ATGGATGAGCGCGTCTGTGATGGTGTCCAGCATGCTCTGC), and 295–335 (CATGCTGTACAGGTGTTCCATGCCTTTGTTGCTCATGTGG) of the 363-bp sequence of midshipman ERα. 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-^{33}P d-ATP (SA, 1000–3000 Ci/mmole; NEN, Boston, MA). Hybridization solution [4× SSC (1× SSC = 0.15 M sodium chloride; 0.015 M sodium citrate, pH 7.2), 40% deionized formamide, 500 μg/ml denatured calf thymus DNA, 250 μg/ml transfer RNA, 4× 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× SCC, washed twice for 30 minutes at 55°C in 1× SCC in a shaking water bath, and washed once in 1× SCC 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-2, 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), counterstained in cresyl violet, dehydrated, and coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ). In situ hybridization for aromatase mRNA was previously published and is similar to that for ERα except a mixture of two oligonucleotides were used for a total radiolabeled probe of 1×10^6 cpm per slide (Forlano et al., 2001). For direct comparison of ERα and aromatase mRNA expression, alternate sections from the same brain of all animals were run through both protocols. The neuroanatomical nomenclature of Braford (1995), Braford and Northcutt (1983), and Northcutt (1995) was adopted for this study.

Immunocytochemistry

Animals (seven females) were perfused and saccular epithelium removed and sectioned as above for ISH. The functional anatomy of the saccular epithelium was delineated with a monoclonal antibody, HCS-1, that specifically labels the cell bodies of hair cells (Gale et al., 2000). A teleost-specific antibody for aromatase was one we developed for our previous study (Forlano et al., 2001). Protocols using teleost-specific aromatase antibodies and double-label fluorescence (see below) follow those from our aromatase study as well (Forlano et al., 2001) and can be summarized as follows: 2 × 10 minutes in 0.1 M phosphate-buffered saline (PBS; pH 7.2), 20 minutes PBS + 1.0% bovine serum albumin (BSA) + 0.3% Triton-X 100, overnight (16 hours) in primary antibody solution [anti-aromatase, diluted 1:5,000; HCS-1 (hair cell-specific antibody, donation of J. Corwin) diluted 1:500 in PBS + 0.5% BSA + 0.3% Triton-X 100 + 0.5% sodium azide], 2 × 10 minutes PBS + BSA, 1 hour in secondary anti-rabbit antibody (aromatase) or anti-mouse (HCS-1) (Vectastain Rabbit IgG kit, Vector Labs, Burlingame, CA) in PBS + BSA, 2 × 10 minutes PBS + BSA, 1 hour Avidin-Biotin Complex (Vectastain Rabbit IgG kit, Vector Labs) in PBS + BSA, 2 × 10 minutes PB, 1.5 minutes diaminobenzidine solution (DAB) [0.05% DAB in 0.1 M PB with 0.3% hydrogen peroxide], 2 × 10 minutes PB, dehydrated in a graded series of ethanol, 3 × 5 minutes xylene, and coverslipped in Permount (Fisher Scientific, Fair Lawn, NJ).

In double-label experiments, both primary antibodies (rabbit anti-aromatase, 1:1,000, and neural-specific anti-Hu protein 1:100 (16A11, Molecular Probes, Eugene, OR) and subsequently both secondary antibodies were combined and applied as above. Secondary anti-rabbit antibody conjugated to fluorescein (Vector) was used to visualize anti-aromatase and secondary anti-mouse antibody conjugated to Texas Red (Vector) was used to visualize anti-Hu. Both secondary fluorescence antibodies were used at 1:100 dilution in PBS + 0.5% BSA. Slides were incubated for 1.5 hours in a humidified chamber, washed 3 × 10 min in PBS, dipped in ddH_2O, and coverslipped with Vectashield (Vector).

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 and fluorescence, iso 100 for brightfield) and a 35 mm Nikon camera mounted on a Nikon ES800 compound microscope. Fluorescently labeled material was viewed on the above microscope outfitted with epifluorescence and a double-band pass cube (FITC/Texas Red) to allow for simultaneous visualization of both antibodies. Negatives were then scanned at 1800 dpi, compiled into plates and, in most cases, converted to grayscale, contrast enhanced, and labeled in Adobe PhotoShop 5.0 (San Jose, CA).

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GTCGAGGGGATGGCAGAAAIATTCGATATGCTGCTGGCTACCACCGCCCGC
V  E  G  M  A  E  I  F  D  M  L  L  A  T  T  A  R

TTCCGCATGCTGAAACTCAACACGGAGGAGTTTGTGTGTCTCAAGGCGATC
F  R  M  L  K  L  N  T  E  E  F  V  C  L  K  A  I

GTCTTACTCAACTCCGGAGCCTTTTCCTTCTGCACCAGCACAATGGAGCCG
V  L  L  N  S  G  A  F  S  F  C  T  S  T  M  E  P

CTGCACGACGGCGCGGCGGIGCAGAGCATGCTGGACACCATCACAGACGCG
L  H  D  G  A  A  V  Q  S  M  L  D  T  I  T  D  A

CTCATCCATCACATCAGCCAATCAGGAGGCCCCATCCAGCATCAGTCCAGA
L  I  H  H  I  S  Q  S  G  G  P  I  Q  H  Q  S  R

CGTCAGGCCCAGCTCCTCCICCTGCTCTCCCACATTCGCCACATGAGCAAC
R  Q  A  Q  L  L  L  L  L  S  H  I  R  H  M  S  N

AAAGGCATGGAACACCTGTACAGCATGAAGTGTAAGAACAAGGTTCCTCTC
K  G  M  E  H  L  Y  S  M  K  C  K  N  K  V  P  L

TACGAT
Y  D
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Fig. 1. Nucleotide and deduced amino acid sequence of ERα partial cDNA clone isolated from midshipman liver (GenBank Accession no. AY466470).

RESULTS

Cloning of partial cDNA for midshipman estrogen receptor alpha

A predicted 363 bp sequence was amplified from female midshipman liver cDNA and was verified from 13 independent clones. Figure 1 shows the midshipman partial cDNA sequence with translated amino acid sequence below (GenBank Accession no. AY466470). This sequence falls within the ligand-binding domain of ERα and is highly conserved in teleosts as well as other vertebrate groups. A comparison with other teleost as well as zebra finch, rat, and human ERα's is shown in Figure 2. A BLAST (National Center for Biotechnical Information) search using the midshipman translated protein revealed a 92% sequence identity to Atlantic croaker, *Micropogonias undulatus* (GenBank Accession no. AF298 183.1), 90% identity to red seabream, *Chrysophrus major* (GenBank Accession no. AB007453), 88% identity to threespot wrasse, *Halichoeres trimaculatus* (GenBank Accession no. AF326201.1), 87% identity to Nile tilapia, *Oreochromis niloticus* (GenBank Accession no. U75604.1), 83% identity to channel catfish, *Ictalurus punctatus* (GenBank Accession no. AF061275.1), 82% identity to rainbow trout, *Oncorhynchus mykiss* (GenBank Accession no. AJ242741.1), 68% identity to zebra finch, *Taeniopygia guttata* (GenBank Accession no. L79911.1), 66% identity with Norway rat, *Rattus norvegicus* (GenBank Accession no. X61098.1), and 66% identity with human, *Homo sapiens* (GenBank Accession no. AF258450.1). Sense and antisense degener-

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midshipman      1   VEGMAEIFDMLLATTARFRMLKLNTEEFVCLKAIVLLNSGAFSFCTSTMEPLHDGAAVQS
croaker       317   VEGMAEIFDMLLATTSRFRMLKLKTEEFVCLKAIILLNSGAFSFCTGTMEPLHDGAAVQN
red seabream  380   VEGMAEIFDMLLATASRFRMLKLKPEEFVCLKAIILLNSGAFSFCTGTMEPLHDGAAVQN
wrasse        259   VEGMAEIFDMLLATTSPFRMLKLKPEEFVCLKAIVLLNSGAFSFCTGTMEPLHDSAPVQD
tilapia       374   VEGMAEIFDMLLATASRFRVLKLKPEEFVCLKAIILLNSGAFSFCTGTMEPLHDSAAVQH
catfish       423   VEGMAEIFDMLLATVARFRTLKLKSEEFVCLKAIILLNSGAFSFCSSPVEPLRDGFMVQC
trout         386   VEGMAEIFDMLLATVSRFRMLKLKPEEFVCLKAIILLNSGAFSFCSNSVESLHNSSAVES
zebra finch   410   VEGMVEIFDMLLATAARFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDYIHR
rat           423   VEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHR
human         418   VEGMVEIFDMLLATSSRFRMMNLQGEEFVCLKSIILLNSGVYTFLSSTLKSLEEKDHIHR

midshipman     61   MLDTITDALIHHISQSGGPIQHQSRRQAQLLLLLSHIRHMSNKGMEHLYSMKCKNKVPLYD
croaker       377   MLDTITDALIHHISQSGCSAQQQSRRQAHLLLLLSHIRHMSNKGMEHLYSMKCKNKVPLYD
red seabream  440   MLDTITDALIHHINQSGCSAQQQSRRQAQLLLLLSHIRHMSNKGMEHLYSMKCKNKVPLYD
wrasse        319   MLDIITDALIHHISQSGCSAHQQSRRQAQLLLLLSHIRHMSNKGMEHLYSMKCKNKVPLYD
tilapia       434   MLDTITDALIFHISHLGCSAQQQSRRQAQLLLLLSHIRHMSNKGMEHLYSMKCKNKVPLYD
catfish       483   MMDNITDALIYYISQSGISVQLQSRRQAQLLLLLSHIRHMSYKGMEHLYSMKCKNKVPLYD
trout         446   MLDNITDALIHHISHSGASVQQQPRRQAQLLLLLSHIRHMSNKGMEHLYSIKCKNKVPLYD
zebra finch   470   VLDKITDTLIHLMAKSGLSLQQQHRRLAQLLLILSHIRHMSNKGMEHLYNMKCKNVVPLYD
rat           483   VLDKITDTLIHLMAKAGLTLQQQHRRLAQLLLILSHIRHMSNKGMEHLYNMKCKNVVPLYD
human         478   VLDKITDTLIHLMAKAGLTLQQQHQRLAQLLLILSHIRHMSNKGMEHLYSMKCKNVVPLYD
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Fig. 2. Alignment of deduced amino acid sequences of midshipman with other teleost (croaker, red seabream, wrasse, tilapia, catfish, and trout), avian (zebra finch), and mammalian ERα's. Shaded areas indicate identical amino acids to midshipman ERα. Underlined flanking sequences indicate conserved regions to which degenerate primers were made for RT-PCR. This sequence falls within the ligand binding domain and is up to 92% conserved with other teleosts.

ate primers for RT-PCR were designed to the flanking underlined amino acid sequences.

RT-PCR and Quantitative real-time PCR

A consistent 303 bp product was seen in all tissues at 25 and 35 cycles. At 25 cycles, qualitative differences in expression by ethidium bromide staining could be seen (liver and ovary greater than other areas) and this was substantiated by quantitative methods (see below). No bands were seen in no-RT or no-template controls. In real-time SYBR Green PCR reactions, amplification of both ERα and β-actin (internal control) products showed consistent threshold cycles (Ct) for triplicate samples. Negative control tubes had Ct of > 37, or nondetectable. Relative levels of ERα mRNA expression were normalized to β-actin control for major brain regions as well as liver, ovary, and sensory epithelium of the inner ear. Since hindbrain samples had the lowest values of any area, values are expressed as fold differences relative to hindbrain. As expected, liver and ovary showed highest levels, 109.54 and 4.66 times greater, respectively, than hindbrain. Forebrain was 2.31-, midbrain 1.96-, and sensory epithelium of the inner ear was 1.92-fold greater than hindbrain.

In situ hybridization

A neuroanatomical atlas of ERα mRNA distribution is presented in Figures 4–6, 8, and 9 at nine transverse levels shown in Figure 3 for the brain from a nesting male (standard length, 15.6 cm) collected during the spawning period in June 2002. For each representative area, the left half of the Nissl-stained section is presented in brightfield (left) and the same section shown in darkfield (right) to

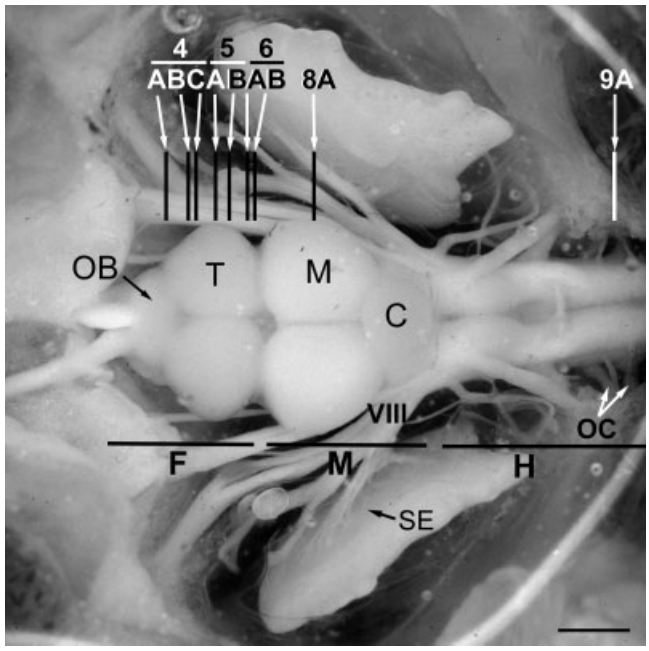

Fig. 3. Dorsal view of exposed brain and inner ear of a midshipman indicating levels (rostral–caudal) of transverse sections for the brain atlas of ERα distribution shown in Figures 4–6, 8, and 9. Underlined areas correspond to forebrain (F), midbrain (M), hindbrain–spinal cord (H) dissected regions used to determine relative mRNA levels by real-time PCR. C, cerebellum; M, midbrain; OB, olfactory bulb; OC, occipital nerve roots; T, telencephalon; SE, saccular epithelium of the inner ear; VIII, eighth nerve. Scale bar = 1.5 mm.

view the most abundant ERα hybridization patterns. Because signal in the midbrain and hindbrain is more diffuse and comparatively weaker than in most forebrain regions, visualization was more effective at higher magnification in the brightfield. The general pattern of ERα distribution shown in the atlas is representative of all other animals used in this study. Certain areas that were clearly labeled in some animals but not others are represented in the atlas by insets and are discussed below. While these differences in signal intensity may yet represent either sex or seasonal differences, the intent here was to provide a comprehensive overview of possible sites of ERα distribution in the brain and the inner ear. No specific hybridization pattern was seen with negative controls (sense probes).

Forebrain. Prominent ERα hybridization signal is first detected in the anterior forebrain, ventral to the olfactory bulb within the ventral nucleus of area ventralis of the telencephalon (Vv; Fig. 4A). In some animals, scattered cells were robustly labeled in the rostral olfactory bulb (Fig. 4A inset, rostral to level of 4A). The highest concentration of ERα-expressing cells in the brain is in the anterior parvocellular preoptic area (PPa, Fig. 4B,C). ERα mRNA is most abundant rostrally, along the ventromedial border of the PPa (Fig. 4B) extending into the anterior commissure (ac), whereas a section through the caudal end of the PPa shows high signal concentrated along the ventricular midline with ventrolaterally clustered cell groups (Fig. 4C). In recrudescent females, caught during the seasonal peak of circulating estradiol (Sisneros et al., 2004a), ERα signal was also detected along the ventricular border of the supracommissural nucleus (Vs), extending into the caudal aspect of the dorsal nucleus (Vd) of area ventralis at the level of the anterior commissure (Fig. 4B, inset). The magnocellular nucleus of the preoptic area (PM, Fig. 5A) is well defined by strong ERα expression throughout its extent, and is apparent at its most caudal region, the gigantocellular division (PMg) (Fig. 5B). Figure 5A also shows ERα mRNA dorsal to PM in discrete regions of the medial division of area dorsalis of the telencephalon (DM). Hybridization signal is most prominent in posterior (DMP) and caudomedial (DMcm) regions of DM at the caudalmost extent of the telencephalon (Fig. 5B); DMcm is continuous with ERα mRNA in a more rostral portion of the central zone of area dorsalis (DC, Fig. 5A). ERα expression within the DMcm-DC continuum extends ~800 μm through the dorsal telencephalon. The ventrolateral border of the posterior division of area dorsalis (DP) also exhibits ERα expression (Fig. 5A,B). Less intense signal was seen covering the caudal portion of the posterior nucleus of area ventralis (VP) (inset, Fig. 5A) and in the ventral tuberal nucleus (not shown). High levels of ERα expression, of comparable intensity to those observed for the PPa, were seen in the ventrolateral nucleus of the hypothalamus (Hv) (Fig. 6A,B). The anterior tuberal nucleus (AT) that lies dorsolateral to Hv also shows ERα expression. Contiguous with AT, and slightly more caudal, is the dorsal periventricular hypothalamus (Hd), another region of high ERα expression (Fig. 6B).

Diencephalon/pineal complex (prespawning female). Although sex differences were not systematically measured in this study, two females caught in the prespawning period undergoing gonadal recrudescence showed intense hybridization in the pineal stalk (PS, Fig. 7A,B) which was not detected in other males or females caught during the spawning period. The pineal organ appears to contain some of the highest densities of ERα mRNA in any brain region. For these specimens, hybridization in other diencephalic areas was apparent as either small grain clusters in the periventricular posterior tuberal nucleus along the ventricular midline (TPp, arrows, Fig. 7A,B) or diffusely distributed along the medial edge of the dorsal posterior nucleus of the thalamus (DPo) (Fig. 7B), which was not obvious in animals collected during the spawning season (compare to Fig. 6).

Brainstem. In all animals, ERα hybridization was consistently found in the nucleus of the medial longitudinal fasiculus (nMLF) along the midline ventral to the periaqueductal gray (Fig. 8A,B). This label was found in discrete clusters along the dorsal and medial tegmental midline throughout most of the rostral midbrain. Labeled cells (not shown) were occasionally found in the optic tectum (TeO) and torus semicircularis (TS) and scattered throughout the tegmentum ventral to the TS. As in other cases, these areas were most noticeable in prespawning females. In two animals (one female, one male), the saccus vasculosus (SV, Fig. 8A for location) showed significant levels of hybridization. Low-intensity signal was apparent in the sonic motor nucleus (SMN) but higher magnification revealed specific clustering over motor neurons and smaller cells along the lateral periphery (Fig. 9A,C).

Pituitary–gonadal axis. The pituitary and ovary essentially served as positive controls. ERα hybridization was robust and specific to areas of the anterior pituitary (Fig. 10A). This pattern was consistent among all animals. Similarly, ovaries from recrudescing females showed ERα expression in follicular and interstitial cells which surround maturing oocytes (Fig. 10B,C). These results were consistent with previous studies in which both pituitary and ovarian tissues were documented to contain ERα in teleosts (Andreassen et al., 2003; Fine et al., 1990; Menuet et al., 2003).

Inner ear. The functional anatomy of the saccule (main organ of hearing in teleosts) in the inner ear was delineated by use of a monoclonal hair cell-specific antibody, HCS-1, which specifically labels the cell bodies of hair cells (Gale et al., 2000) (Fig. 11A–D). This proved invaluable to identify the orientation of ERα localization in the inner ear as the angle of sectioning varied between samples. ERα mRNA was consistently found in eighth nerve branches that innervate the hair cell layer of the saccular epithelium (Fig. 11E,F) and less often seen scattered throughout the eighth nerve distal to the epithe-

Fig. 4. Distribution of ERα mRNA in the ventral telencephalon and anterior preoptic area. In this figure and in 5–6 and 8–9, neuroanatomical localization of in situ hybridization is shown for the same section in brightfield Nissl-stained sections to the left and in darkfield to the right. **A:** Hybridization signal along the ventrolateral border of Vv (ventral nucleus of area ventralis). A, inset: Scattered punctate hybridization signal throughout the rostral ventral olfactory bulb. **B:** ERα mRNA expression is greatest at the level of the rostral anterior parvocellular preoptic area (PPa), especially the medial border extending into the anterior commissure (ac). B, inset: At this level, mRNA is more apparent along the dorsomedial border of the supracommissural (Vs), extending into the dorsal (Vd) nucleus of area ventralis (designated by asterisk) in a prespawning female. **C:** Hybridization signal in the caudal PPa lining the ventricle just below the anterior commissure and in ventrolaterally clustered cell groups. Scale bars = 500 μm for all except A, inset = 200 μm.

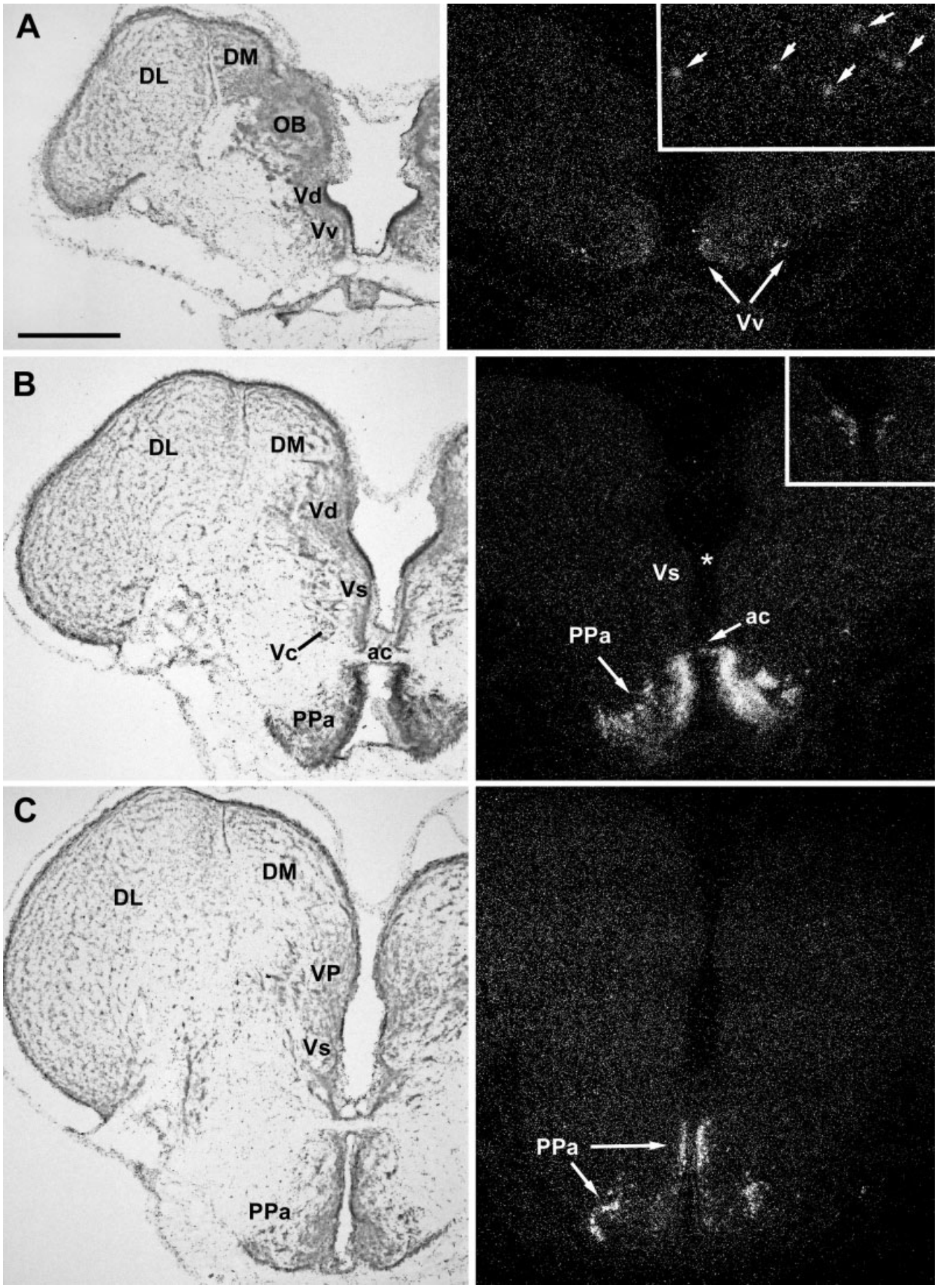
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Figure 4

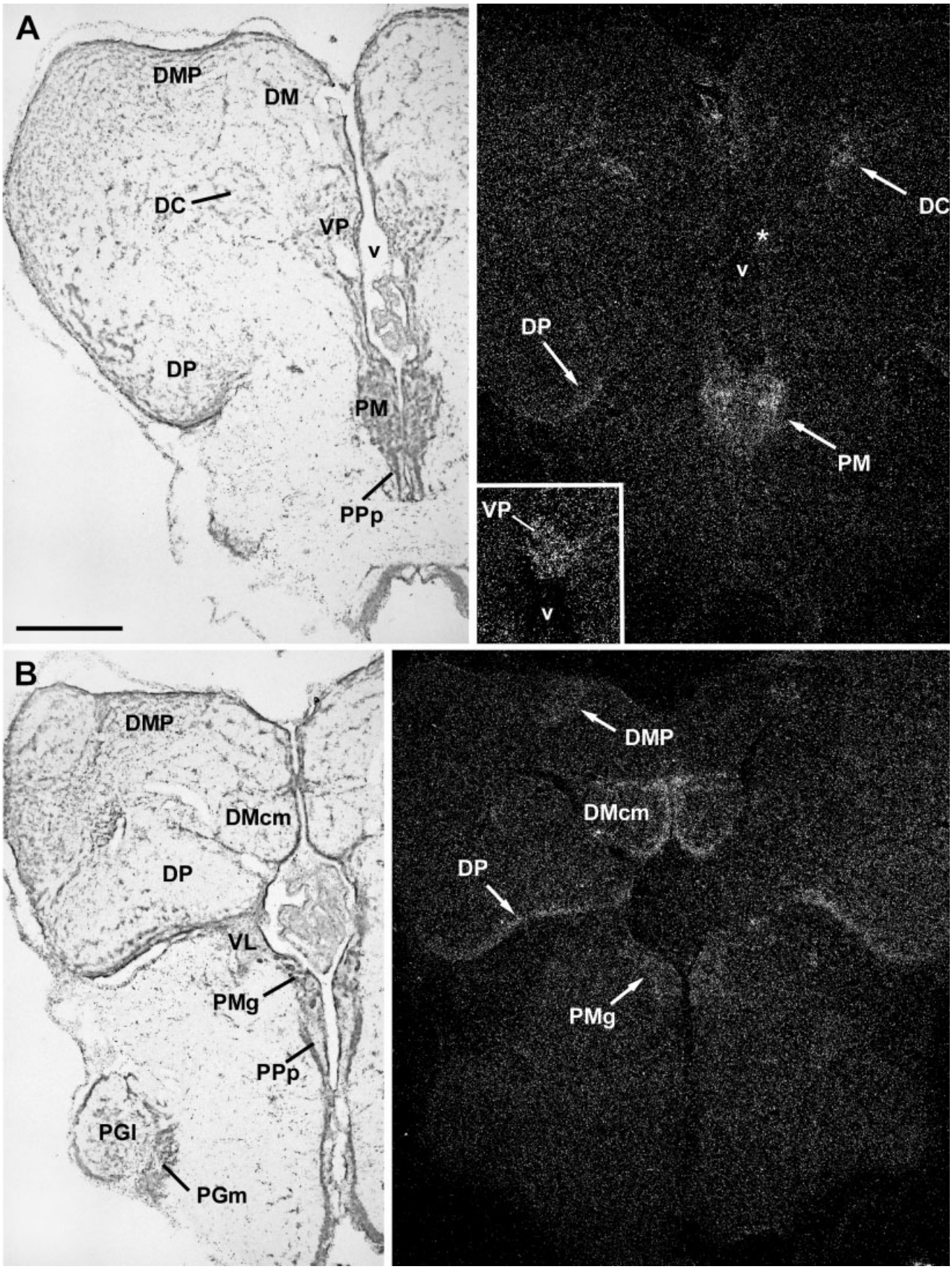

Fig. 5. Distribution of ERα mRNA in the dorsal telencephalon and caudal preoptic area. **A,B:** Hybridization signal is seen throughout the extent of the magnocellular preoptic nucleus, but is especially strong in its anterior magnocellular division (PM); lighter signal appears in the gigantocellular division of PM (PMg). No obvious signal was detected in the posterior parvocellular preoptic nucleus (PPp). ER mRNA is also localized in discrete areas in the dorsal pallium including DC (central zone of area dorsalis), the ventrolateral border of DP (posterior zone of area dorsalis), and DMP and DMcm (posterior and central medial divisions of the medial zone of area dorsalis). A, inset: Visible signal in the postcommissural nucleus of area ventralis (VP, indicated by asterisk) of a prespawning female. Scale bar = 500 μm.

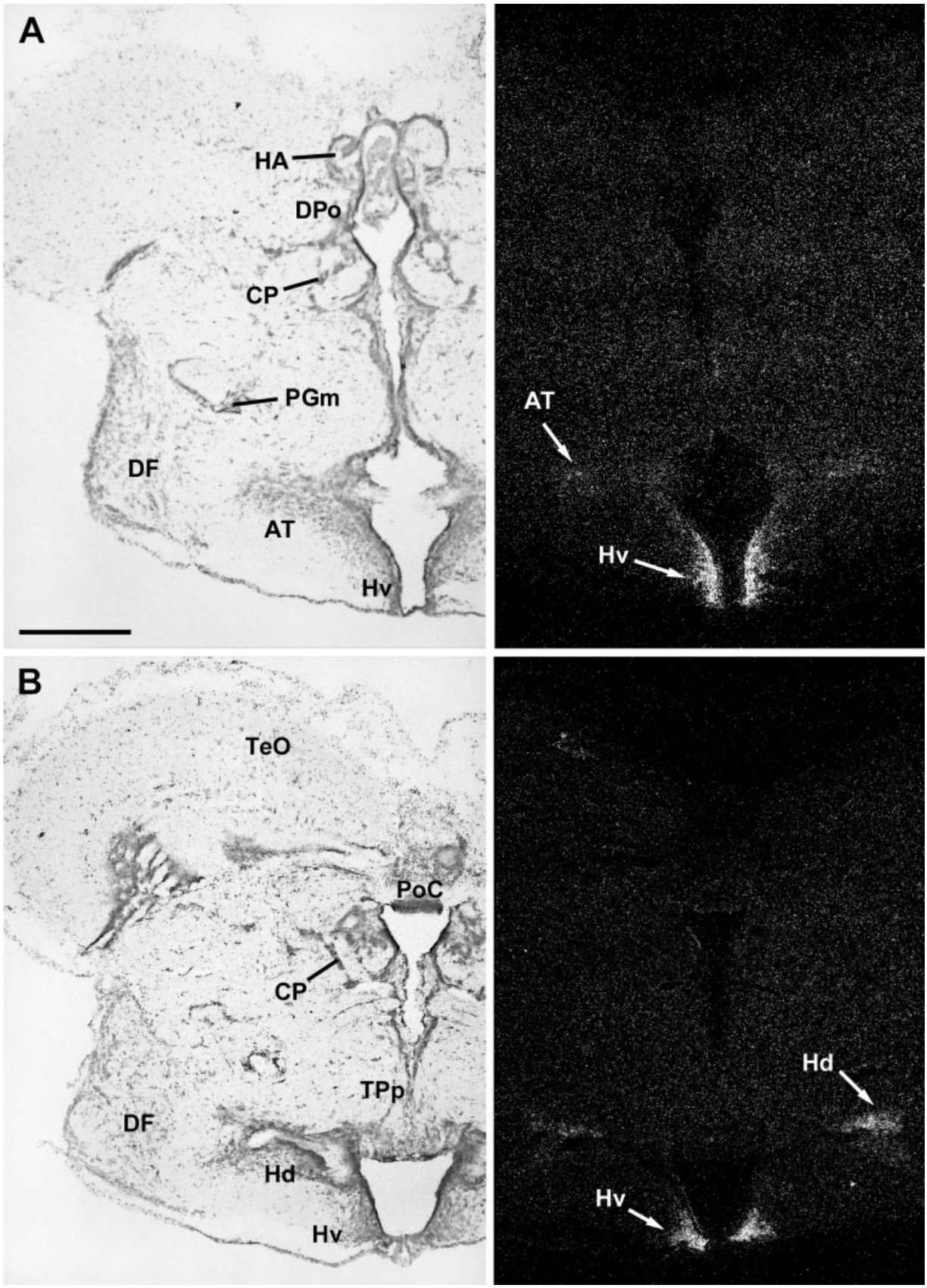

Fig. 6. Distribution of ERα mRNA in the hypothalamus. **A,B:** High levels of ERα mRNA in the ventrolateral (Hv) and dorsal periventricular (Hd) nucleus of the hypothalamus, as well as in the anterior tuberal nucleus (AT), part of the forebrain vocal-acoustic center. Scale bar = 500 μm.

lium. There was no consistent robust signal over ganglion cells or in the hair cells themselves as seen over the eighth nerve. Like ERα expression in the brain, expression in the ear was more apparent in prespawning females undergoing gonadal recrudescence during which a seasonal peak of estradiol is known to occur (Sisneros et al., 2004a).

Comparisons to aromatase distribution

In order to directly compare sites of estrogen receptor expression to estrogen synthesis in the brain, we examined alternate sections hybridized to midshipman-specific aromatase mRNA probes (see Forlano et al., 2001). Figure 12 shows several levels of aromatase mRNA distribution throughout the forebrain. As reported previously, the telencephalon has the most abundant levels of brain aromatase activity (Schlinger et al., 1999) and protein and mRNA expression (Forlano et al., 2001). Aromatase mRNA has a broad distribution and is found concentrated in preoptic areas along the ventricular midline as well as the entire peripheral surface of the telencephalic hemispheres (also ventricular surfaces in teleosts). This unique distribution pattern of aromatase mRNA was revealed to be concentrated in radial glia whose somata line the ventricle and whose processes extend and cover virtually the entire telencephalon (see Forlano et al., 2001). Thus, at least in the forebrain, aromatase is largely ubiquitous. Aromatase mRNA has an identical distribution pattern to the protein, so, in general, the highest levels shown by in situ hybridization correspond to glial somata lining the ventricle, while the more diffuse signal seen centrally corresponds to the broad distribution of aromatase in glial fibers (Forlano et al., 2001).

Rostrally, dense concentrations of aromatase mRNA have a similar distribution pattern to ERα in the anterior preoptic area, anterior commissure (PPa, ac, Fig. 12A, compare to Fig. 4B), and medial edge of the supracommissural nucleus (Vs, Fig. 12A, compare to Fig. 4B, inset). While grain clusters indicative of ERα mRNA expression are scattered within the central region of the rostral olfactory bulb (Fig. 4A, inset), aromatase-immunoreactive (ir) cells are concentrated along the periphery of the bulb (see Forlano et al., 2001). In the caudal preoptic area, aromatase mRNA is highest along the ventricular midline in magnocellular (PM) and posterior parvocellular (PPp) divisions, unlike ERα, which is concentrated throughout PM but mostly absent from PPp (compare Figs. 12B, 5A,B). Similarly, aromatase is abundant along the ventricular border of VP (posterior nucleus of the ventral telencephalon), whereas ERα, if present, is diffuse throughout this nucleus (compare Figs. 12B, 5A, inset). Both aromatase and ERα are found along the ventrolateral border of DP, whereas aromatase is more apparent in the ventral tuberal nucleus (VT) (compare Figs. 12B, 5A). Although the area dorsalis is covered by diffuse aromatase mRNA, hybridization signal did not appear to be concentrated centrally in specific nuclei as seen in ERα (i.e., DC, DMP, DMcm), although aromatase was concentrated in the caudalmost telencephalon containing DMcm (compare Figs. 12C, 5B).

Figure 12C,D shows aromatase mRNA distribution in the diencephalon in relation to adjacent sections labeled for ERα mRNA in Figure 7A,B, respectively. In the diencephalon, aromatase is most abundant along the third ventricle, from the dorsal posterior and central posterior nuclei of the thalamus (DPo, CP) and the periventricular posterior tuberal nucleus (TPp) to the prominent paraventricular organ (PVO) and dorsal and ventral periventricular nuclei of the hypothalamus (Hd, Hv). From the center midline, aromatase mRNA distribution extends laterally, which corresponds to glial processes originating from the ventricle (Forlano et al., 2001). In contrast, ERα is concentrated in the periventricular hypothalamus, but only diffusely in the TPp and the dorsal thalamus (DPo). Both ERα and aromatase are found in the anterior tuberal nucleus (also see Fig. 6A). Like ERα, aromatase was only found in the pineal stalk in recrudescing females, although ERα is comparatively more robust in this area.

In the midbrain, aromatase mRNA is concentrated medially along the periaqueductal gray (PAG) and in the nucleus of the medial longitudinal fasciculus (nMLF) (Fig. 8C,D). A more diffuse signal (corresponding to aromatase-containing glial fibers) extends ventrolaterally to cover most of the ventral tegmentum. In contrast, ERα is largely restricted to the nMLF (compare Fig. 8D to 8B, which are adjacent sections). Aromatase is absent in the optic tectum and torus semicircularis, whereas ERα mRNA is occasionally found in scattered cells in both of these areas as well as in areas of the ventral tegmentum covered by aromatase. In the hindbrain-spinal cord, aromatase mRNA enshrouds the sonic motor nucleus. Aromatase-containing glial cells line the dorsal aspect of the nucleus near the ventral edge of the fourth ventricle and weave their processes around the motor neurons (Forlano et al., 2001). Darkfield visualization of aromatase mRNA distribution demonstrates this pattern that outlines individual motor neurons (Fig. 9B). In contrast, ERα hybridization signal is clustered over the motor neurons themselves (Fig. 9C).

Both the pituitary (mainly anterior, aPit, Fig. 10A) and ovarian tissue (Fig. 10B,C) express aromatase (not shown, but see Forlano et al., 2001 for ovary) and ERα, although ERα in the pituitary is relatively more abundant and restricted to specific regions.

In the inner ear, aromatase-containing ganglion cells are found in the eighth nerve proximal to the saccular epithelium (Fig. 11G,H), while ERα mRNA is found in the nerve branches adjacent to the hair cell layer (Fig. 11E,F).

DISCUSSION

Recent studies which have localized ERα mRNA in teleosts have solely described expression in the core neuroendocrine regions of the forebrain, i.e., anterior preoptic area (PPa) and the ventrolateral hypothalamus (Hv), and in some cases in the posterior tuberal nucleus just dorsal to Hv (Andreassen et al., 2003; Hawkins et al., 2000;

Fig. 7. Distribution of ERα mRNA in the diencephalon of a prespawning female undergoing gonadal recrudescence, a time of the year when females have highest levels of circulating estradiol (see text; also compare to Fig. 6, which are sections from a male collected during the spawning season). **A,B:** Intense levels of ERα mRNA expression are seen throughout the pineal stalk (PS) and in the periventricular hypothalamus. Hybridization signal is also apparent along the midline ventricle in the periventricular posterior tuberal nucleus (TPp; arrows), dorsal posterior thalamus (DPo) (lower arrow, B) and in the dorsal pallium (DMP and DMcm; posterior and central medial divisions of the medial zone of area dorsalis). Scale bar = 500 μm.

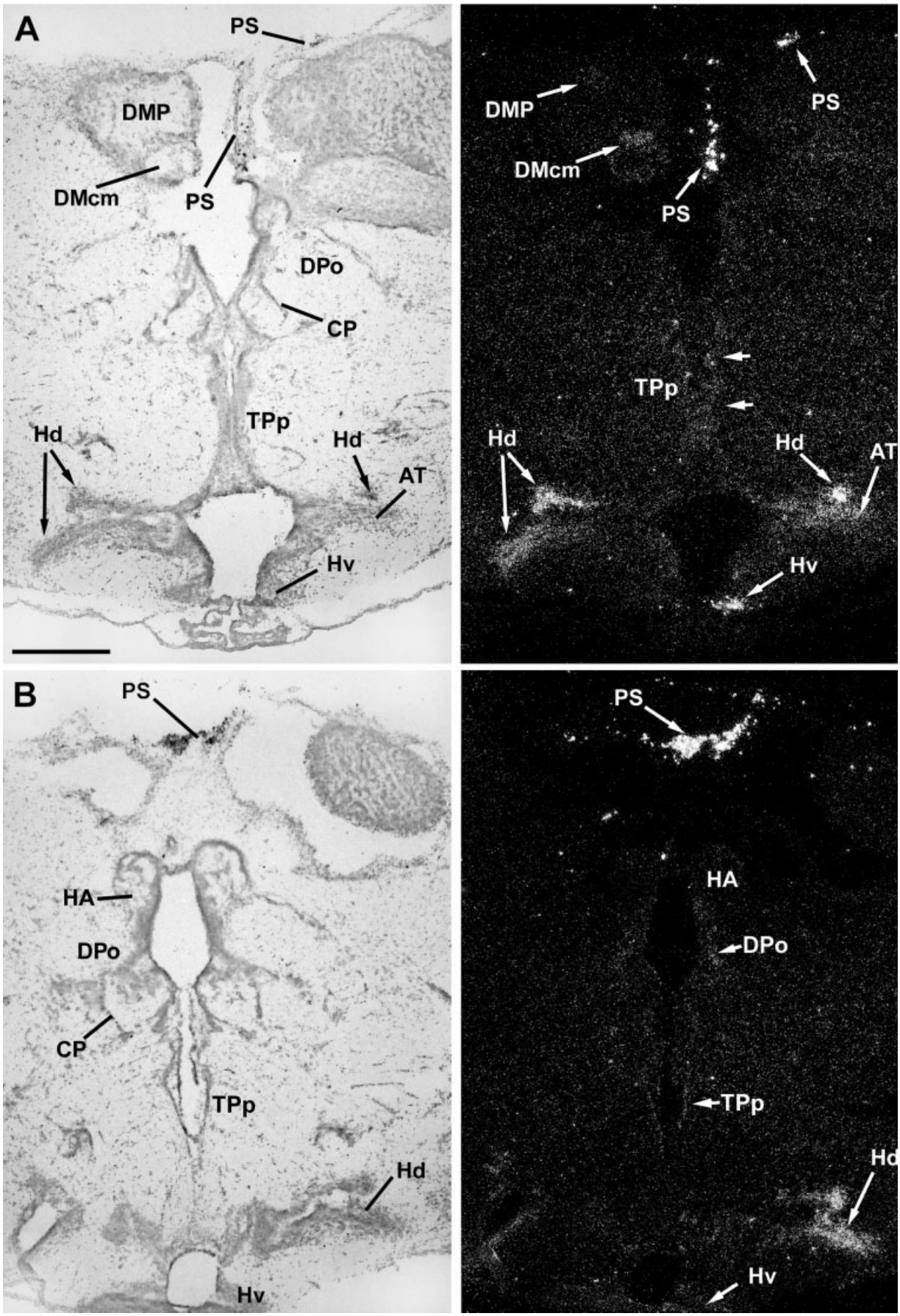
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Figure 7

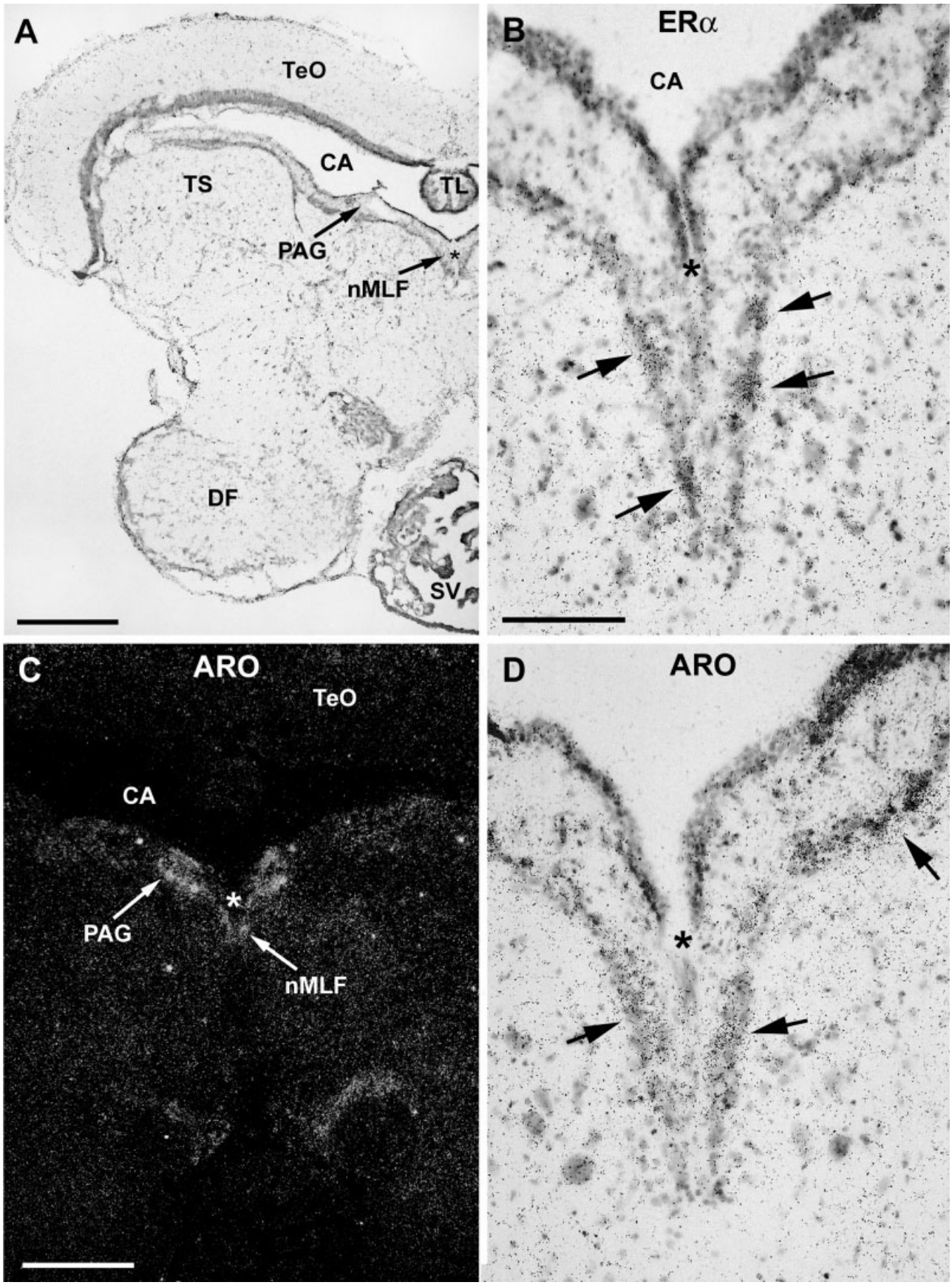

Figure 8

Menuet et al., 2001, 2002). We too observe ERα mRNA in these same regions in midshipman brain, but we also document novel areas of ERα mRNA expression in area dorsalis of the telencephalon, (DC-DMcm, DMP, DP), the midbrain (nMLF), the hindbrain-spinal cord (SMN), the pineal, and in the saccular endorgan of the inner ear.

Earlier studies in teleosts demonstrated [^{3}H] estradiol-concentrating cells consistently in preoptic-Hv regions with interspecies variation outside those regions (Davis et al., 1977; Fine et al., 1990; Kim et al., 1978, 1979). A single study using antibodies specific to the hormone and DNA binding domain of rainbow trout localized ER-immunoreactive cells in the rainbow trout brain in PPa, Hv, and Vv (Anglade et al., 1994). Although cells in area ventralis of the telencephalon (Vv and Vs), the dorsal thalamus, and the midbrain's torus semicircularis were described as [^{3}H] estradiol-concentrating in other species (Fine et al., 1990), the current study is also the first to show ERα mRNA for these areas.

Hybridization signal in Vs and VP was observed only in females collected during a seasonal peak of circulating 17β-estradiol and testosterone (Sisneros et al., 2004a), while expression in DP was consistently found in all animals regardless of sex or time of capture. Although variation in ERα expression in certain brain regions was not unexpected, this study was not designed to compare differences between sexes or seasons. Induction of ERα mRNA after estradiol treatment in teleosts is well documented in the liver (Bowman et al., 2002; Flouriot et al., 1997; MacKay et al., 1996; Pakdel et al., 1991; Sabo-Attwood et al., 2004) and in brain (Sabo-Attwood et al., 2004; Salbert et al., 1993) and may explain why females with high circulating estradiol levels showed expression in areas not seen in other fish that were sampled during times of low circulating gonadal steroids. In fact, all of the above studies which recently described ERα mRNA in the teleost brain by in situ hybridization used fish which were estradiol-treated to maximize detection of the receptor.

Our results show striking conservation in ERα distribution between fishes and mammals (Table 1; also see Davis and Moore, 1996; Jacobs et al., 1996; Rhen and Crews, 2001, for other vertebrate groups). As expected, ERα mRNA was found in preoptic areas (PPa, PM) and ventral hypothalamic areas, as in every other study to date. The preoptic area-anterior hypothalamus (POA-AH) is a conserved area in all vertebrates that expresses steroid receptors and is broadly involved in reproductive cycling and behaviors (for review, see De Vries and Simerly, 2002).

Unexpectedly, we discovered prominent ERα mRNA in the dorsal pallium homolog (area dorsalis, see Braford, 1995; Northcutt, 1995; Wulliman and Mueller, 2004) in all males and females used in this study. Although this is the first demonstration of ERα mRNA expression in the dorsal pallium of a teleost, expression in this region is consistent with ERα in the amygdala of mammals, the proposed homolog of regions of DM (Table 1). A recent study by Portavella et al. (2004) demonstrates that this area of the brain is involved in retention of a conditioned avoidance response in goldfish and lesions to this area causes effects similar to those produced by amygdalar lesions in mammals. Similarly, areas in the ventral pallium homolog (area ventralis, see Braford, 1995; Northcutt, 1995; Wulliman and Mueller, 2004) which receive olfactory input express ERα mRNA (Vv, Vs, VP) and are homologous to the lateral septum, bed nucleus of the stria terminalis, and basal amygdala, respectively, in mammals, which also receive olfactory projections and express ERα mRNA (Table 1). DP, the main target of the olfactory bulb in teleosts, is also a site of ERα mRNA expression like the main olfactory bulb target in the pallium of mammals (piriform cortex, Table 1).

Functional significance of ERα expression

The remainder of the discussion will focus on the possible functional significance of the pattern of ERα mRNA expression recognized here for midshipman fish.

Pineal

During the summer months, the plainfin midshipman seasonally migrates from deep offshore waters to spawn in the intertidal zone along the Pacific Northwest coast of North America. Males build nests, acoustically court females, and care for the young, while females localize male nests by sound, spawn, and return to deep waters offshore. Thus, the timing of reproductive state, as well as vocal signal production and perception, are essential to the reproductive success of this species (Bass and McKibben, 2003; Sisneros et al., 2004a,b). Annual cycles of reproduction in fish are controlled, in part, by the pineal organ (Zachmann et al., 1992). Only females caught during the prespawning period (spring) showed abundant ERα mRNA in the pineal. Thus, ERα may function to link seasonal cues such as changes in day length with timing of migration and gonadal recrudescence. Steroid receptors have been documented in the pineal of other species including androgen receptor (AR) and ERβ in rat (Gupta et al., 1993; Shughrue et al., 1997), AR and ER in human (Luboshitzky et al., 1997), AR in roughskin newt (Davis and Moore, 1996), and ER in trout by Northern blot (Begay et al., 1994). The pineal functions by transducing changes in photoperiod to the neuroendocrine system through an increase in the release of the hormone melatonin during the dark cycle. Steroids, including estradiol, are known to modulate melatonin release from pinealcytes in vitro (Begay et al., 1994; Martin et al., 1996) and other studies have demonstrated that an increase in melatonin (via decrease in day length or melatonin implants) downregulates estrogen receptor expression in the forebrain of mammals (Hill et al., 1996; Lawson et al., 1992; Roy and Wilson, 1981). As stated previously, these females were collected at the time of year when circulating steroid levels are highest. Thus, high levels of circulating estradiol which are known to upregulate ERα may func-

Fig. 8. Distribution of ERα and aromatase mRNA in the midbrain. **A:** Nissl-stained section indicating location of ERα mRNA along the midline nucleus of the medial longitudinal fasiculus (nMLF; asterisk). **B:** High-magnification brightfield micrograph of ERα mRNA (arrows) just below the cerebral aqueduct in the nMLF. Asterisks in A and B are to corresponding locations. **C:** Low-magnification darkfield micrograph showing aromatase mRNA visualized by in situ hybridization. Aromatase is abundant along the periaqueductal gray (PAG) as well as in the nMLF and extends ventrolaterally to cover most of the tegmentum. Signal is absent from the optic tectum (TeO) and torus semicircularis (see TS in A). **D:** High-magnification of nMLF and PAG in C shown in brightfield. Section is adjacent to B. Asterisks in B and D are corresponding locations. Notice similar localization of aromatase in nMLF but much more robust in the PAG (arrows). Scale bars = 500 μm in A; 100 μm in B and D; 400 μm in C.

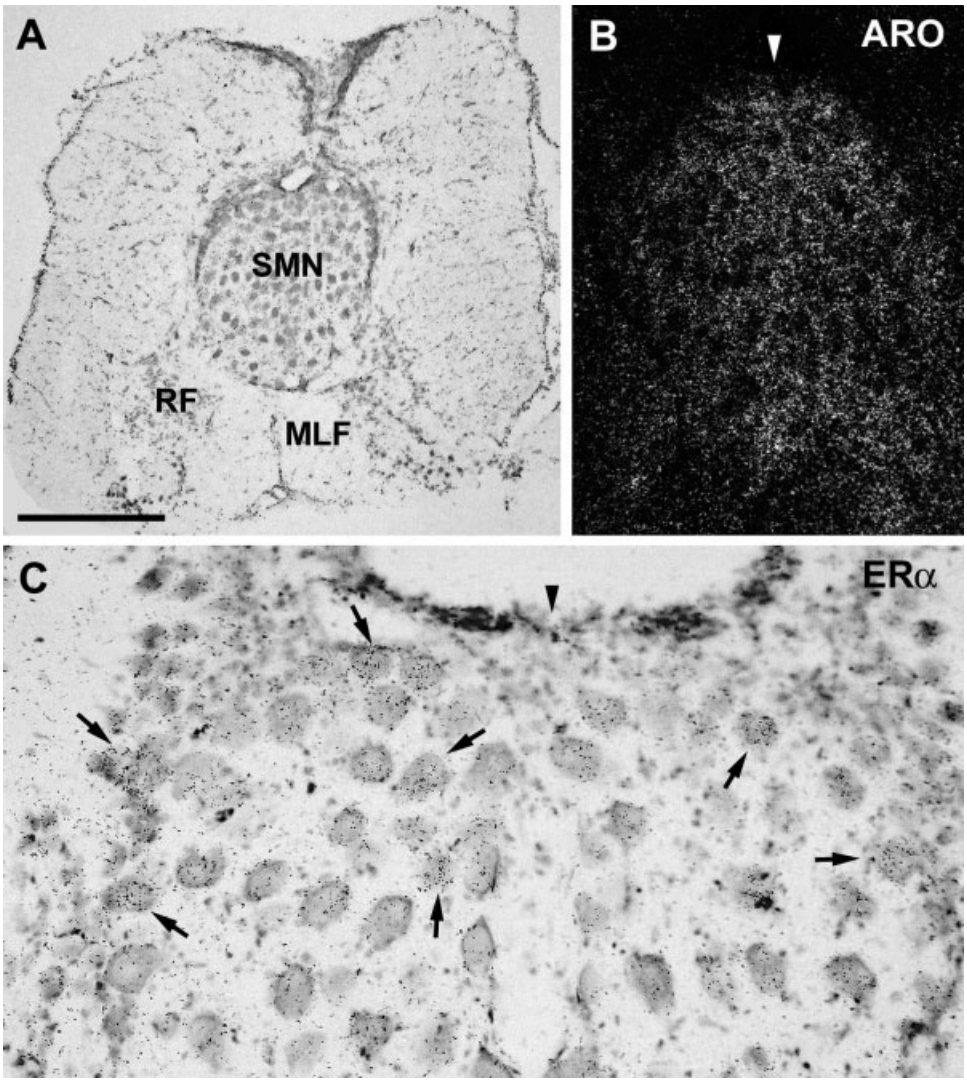

Fig. 9. Distribution of ERα and aromatase mRNA in the sonic motor nucleus (SMN). **A:** Nissl-stained section showing the paired SMN that are contiguous along the midline. **B:** Darkfield micrograph of aromatase mRNA visualized by in situ hybridization. Aromatase enshrouds the SMN but is not expressed in motor neurons (see Forlano et al., 2001) which are outlined by the hybridization signal (round areas absent of silver grains). Arrowhead indicates dorsomedial extent of SMN. **C:** In contrast to aromatase expression, ERα mRNA signal is found clustered over motor neurons in the SMN (arrows) and smaller cells along the lateral periphery (leftmost arrow). Arrowhead indicates dorsomedial extent of SMN. Scale bars = 500 μm in A; 200 μm in B; 100 μm in C.

tion to modulate the rhythmic release of melatonin from the pineal. Expression of steroid receptors in the pineal may occur in males as well, although males caught during the equivalent time of year were not used in this study, and unlike females, circulating sex steroids in males do not peak until the beginning of the summer spawning period (Sisneros et al., 2004a).

ERα expression in relation to brain aromatase

The cellular distribution of aromatase in the brain of teleosts was first investigated in midshipman using teleost-specific antibodies and mRNA probes which revealed localization to glial cells (Forlano et al., 2001). This same pattern was later identified in rainbow trout (Menuet et al., 2003). The distribution of aromatase in the telencephalon is a reflection of its expression in radial glial cells whose somata line the entire periphery of each hemisphere (i.e., ventricular surface) and whose processes extend throughout the entire telencephalon (Forlano et al., 2001). Aromatase-containing glial cells also line the third ventricle and have processes extending throughout the diencephalon (Forlano et al., 2001). Thus, most of the forebrain is potentially bathed in neuroestrogens which may act mainly in a paracrine-like fashion throughout the teleost brain, especially if aromatase and ERα are expressed in different cell types. Neuroestrogens may act in a similar manner in focal regions of the midbrain and hindbrain-spinal cord, including the vocal motor nucleus, where aromatase expression is especially intense (Forlano et al., 2001; also see below).

Several studies have documented that brain aromatase is upregulated by estradiol (Gelinas et al., 1998; Kishida and Callard, 2001; Kishida et al., 2001; Lee et al., 2000) and the teleost aromatase gene contains an estrogen response element (Tchoudakova et al., 2001); therefore, ER in aromatase-containing glial cells would be predicted. Menuet et al. (2003) revealed by RT-PCR that glial cells from primary brain cultures of mature trout do express weak ERα. Expression of ER in glial cells has been documented in the adult and developing mammalian brain (Azcoitia et al., 1999; Jordan, 1999 (review); Donahue et

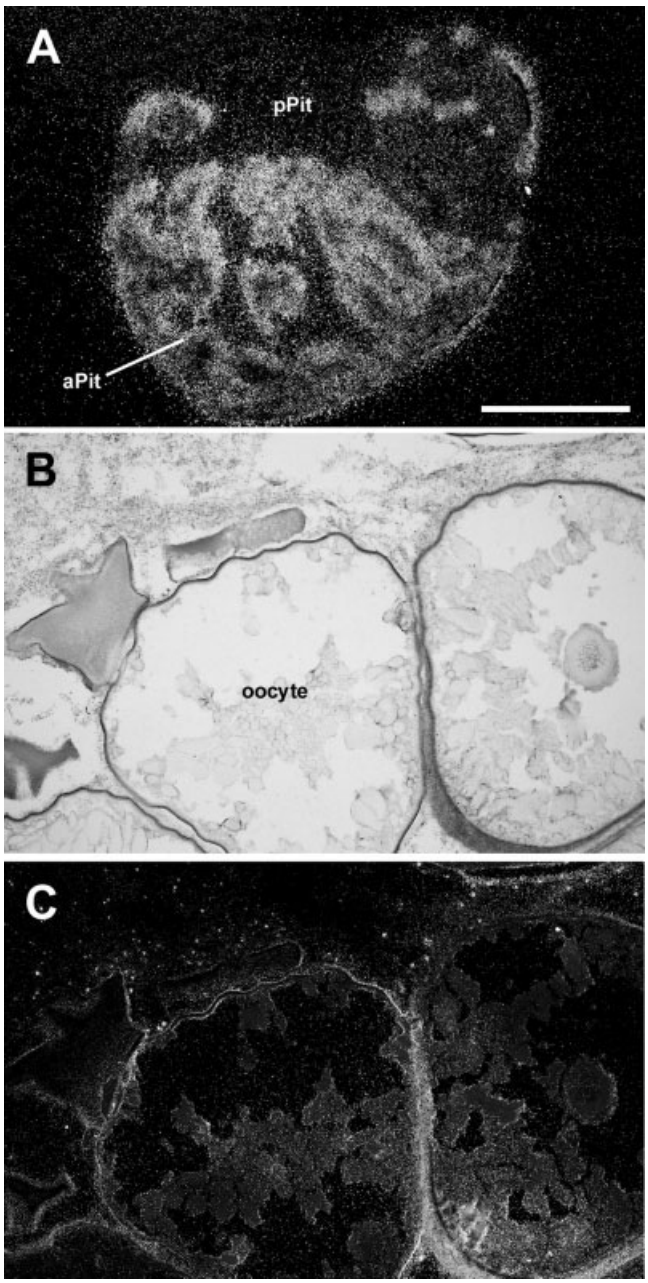

Fig. 10. Distribution of ERα in the pituitary–gonadal axis. **A:** Darkfield micrograph demonstrates robust hybridization in discrete areas of the anterior pituitary. **B,C:** Brightfield and darkfield micrographs showing ERα expression in the follicular and interstitial layers in the ovary of a female undergoing gonadal recrudescence. Scale bars = 250 μm in A; 800 μm for B,C.

al., 2000; Kruijver et al., 2002; Platania et al., 2003; also see Menuet et al., 2003, for more discussion). Since the cellular localization of ERα cannot be established by in situ hybridization alone, teleost-specific antibodies for ERα or more sensitive molecular techniques may be necessary to resolve this issue in situ.

Double-label immunofluorescence revealed aromatase enzyme in ganglion cell bodies and fibers in the auditory nerve proximal to the sensory epithelium of the sacculus. This label was very robust and, unlike the central nervous system, aromatase was found in neurons, not glia. Although aromatase-positive fibers were previously documented in the vestibulocochlear nerve of the neonatal rat (Horvath and Wikler, 1999), this is the first report of aromatase-ir neuronal somata and processes in the auditory nerve of an adult vertebrate. The significance of these findings may reside, in part, in the developmental origin of glial cells and auditory ganglion cells from outside the neural tube, i.e., from either neural crest (glial cells) or placodes (auditory ganglion cells) (Gilbert, 2000).

Although clear co-regionalization and similar patterns of expression of ERα and aromatase are found in the anterior preoptic area and ventricular hypothalamus of midshipman, and ERα expression is comparatively weaker in the midbrain and sonic motor nucleus, ERα does not have the broad distribution of aromatase but rather is concentrated in specific nuclei. This difference is best exemplified by ERα in discrete central zones of the dorsal pallium (DC-DMcm). Conversely, areas such as the paralemiscal/paratoral region in the midbrain, part of the midbrain vocal-acoustic complex (Goodson and Bass, 2002), contain high amounts of aromatase but little detectable ERα. These and other sites in which aromatase is expressed but ERα is not may be indicative of locally produced estrogen acting through either a nongenomic pathway or other ER isoforms. Three separate ER subtypes were first isolated in a teleost, the Atlantic croaker (Hawkins et al., 2000), and have now been described in several other species (Legler et al., 2002; Sabo-Attwood et al., 2004). Thus, the expression of multiple ERs in brain is likely to be widespread among teleosts and may enable a diversity of signaling pathways complementary to high brain aromatase levels.

ERα and aromatase distribution in relation to other enzymes

Based on previous studies in this laboratory, we also recognized several areas in the midshipman brain where ERα and aromatase distribution significantly overlap with other prominent enzymes, namely, choline acetyltransferase (ChAT) and tyrosine hydroxylase (TH). Brantley and Bass (1988) revealed ChAT-ir somata and fibers throughout the SMN, a site of intense aromatase expression (Forlano et al., 2001) and moderate ERα mRNA expression (this report). A second potential site of overlap between aromatase, ERα mRNA and ChAT-ir somata is in Vv (see above references). Several studies have shown that estrogens regulate cholinergic neurons in the basal forebrain of rodents which are important in cognitive function. Estrogen induces ChAT activity in neurons of the basal forebrain which project to the cortex and hippocampus, and it is suggested that estrogen has a trophic effect on these neurons (for review, see McEwen, 2001). Also, ERα is the predominant ER localized in cholinergic neurons in the basal forebrain (Shughrue et al., 2000), and natural changes in ChAT mRNA expression follow the cyclicity of the menstrual cycle and fluctuations in serum estradiol levels (Gibbs, 1996). Furthermore, ERα mRNA in the zebra finch brain appears to overlap a population of sexually dimorphic cholinergic neurons in the ventral paleostriatum that project to song nuclei (Perlman and Arnold, 2003; Sakaguchi et al., 2000). Thus, based on co-regionalization of aromatase and ChAT in midshipman

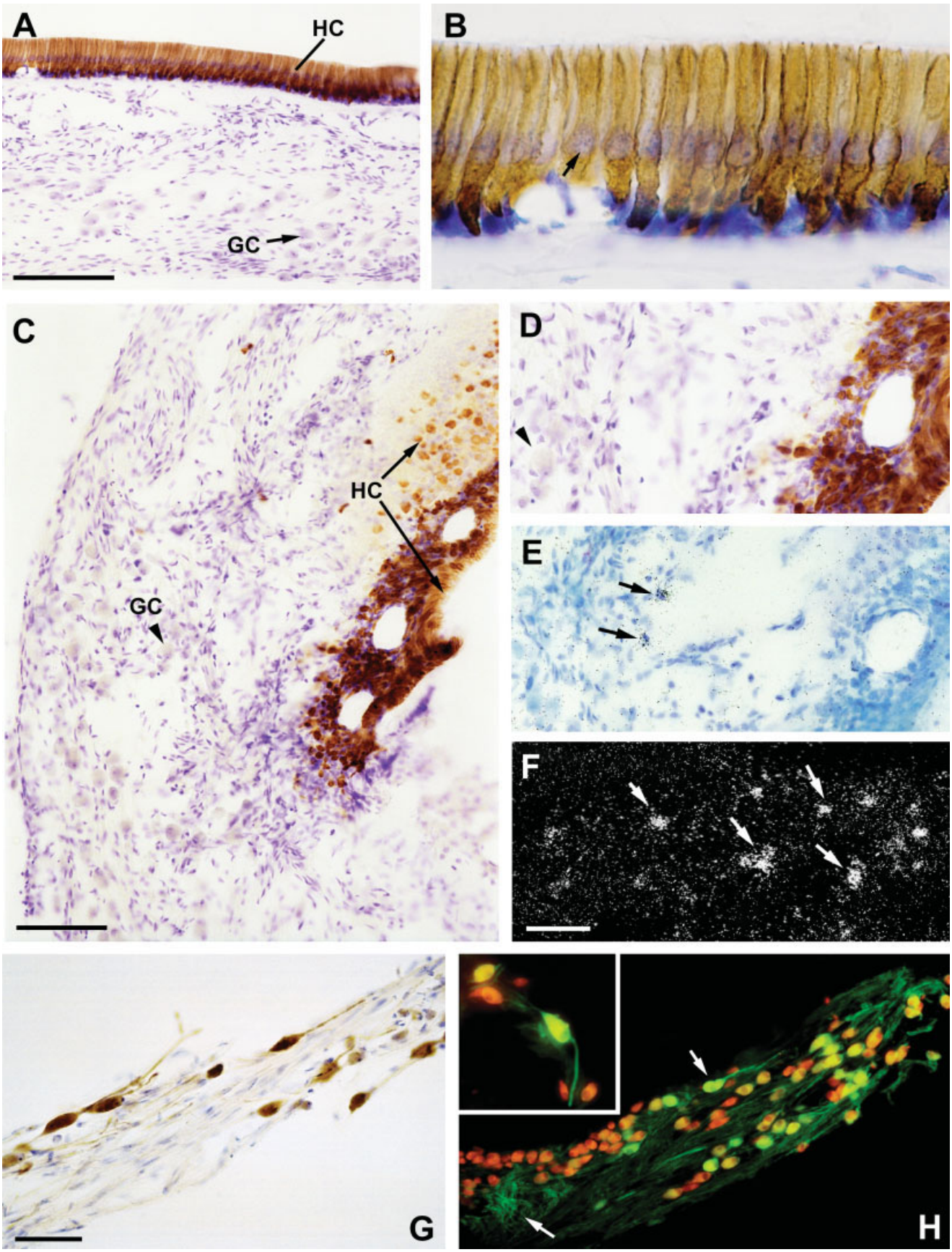

Figure 11.

brain, the hypothesis that estradiol may regulate cholinergic activity through ERα or other ERs deserves further investigation, especially in relation to neurons involved in vocalization.

The distribution of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis was investigated in the midshipman brain and found to innervate auditory and vocal motor nuclei (Bass et al., 2001). High aromatase and ERα expression overlap with TH-ir somata in the PPa, areas ventralis and olfactory bulb in the telencephalon, ventral thalamus (TPp and PVO) and hypothalamus, and dense TH-ir terminals were in the SMN and vocal-acoustic hypothalamus (AT). Neuroanatomical evidence for colocalization of ER and TH was found in the anterior preoptic area of rainbow trout which is thought to control negative feedback of estradiol on gonadotropin secretion (Linard et al., 1996). Another study by Vetillard et al. (2003) demonstrated regulation of TH expression by estradiol in the PPa but not in the olfactory bulb of rainbow trout. Although TH-ir cells have a large distribution throughout the brain of this species (Vetillard et al., 2002), regulation by estradiol outside these areas was not investigated. Studies in avian models (Japanese quail and songbirds) have provided evidence that catecholamines serve as a link between steroids and their targets (for review, see Absil et al., 2001). In quail, for example, although aromatase in the preoptic area is regulated by testosterone and estradiol, ERs are not generally colocalized in this region of the brain. However, aromatase-containing neurons are innervated by steroid sensitive TH-ir (and arginine vasotocin-ir) fibers and terminals and thus aromatase activity could be regulated transsynaptically. Additional in vivo and in vitro experiments provided evidence for direct control of aromatase by catecholamines which could occur by transcriptional or nongenomic mechanisms. Evidence exists for ERα mRNA in TH-ir nuclei in the brainstem of canaries which project to song control centers in the forebrain. Therefore, steroids could affect song production by modulating catecholaminergic inputs (Ball et al., 2003). Thus, the presence of aromatase and/or ERα in TH-ir areas in the midshipman brain suggests another pathway in which neuroestrogens could modulate vocal-acoustic physiology and behavior.

Central vocal and auditory systems

Expression of ERα was found in forebrain (PPa, AT) nuclei that are sites of integration between the auditory and vocal motor systems (Bass et al., 1994, 2000; Goodson and Bass, 2002) (see Table 1). This point is highlighted in Figure 13A, which shows inputs to both AT and the central posterior nucleus (CP) following a neurobiotin injection into a more anterior positioned vocally active site in the rostral hypothalamus, the ventral tuberal nucleus (from Goodson and Bass, 2002; AT and CP also receive direct input from the midbrain auditory center in the torus semicircularis; Bass et al., 2000). The AT is also densely innervated by the neuropeptides arginine vasotocin (AVT) and isotocin (as revealed by an antibody to the closely related oxytocin peptide, Fig. 13B; Goodson and Bass, 2000b; Goodson et al., 2003) which modulate vocal-motor patterning in midshipman fish (Goodson and Bass, 2000a). Arginine vasotocin and its mammalian homolog arginine vasopressin are known modulators of vocal-motor systems across a range of vertebrate groups (Emerson and Boyd, 1999; Goodson and Bass, 2001). Figure 13C also demonstrates robust ERα mRNA expression throughout AT in a prespawning female (compare to oxytocin-like immunoreactivity in Fig. 13B). Based on overlap in localization, we hypothesize that ERα modulates neuropeptide expression in this area as estradiol (along with androgens) is known to affect AVP expression in limbic regions (amygdala, BnST) in mammals (De Vries et al., 1986, 1994; Scordalakes and Rissman, 2004) and amphibians (Boyd, 1997), and aromatization of T to estradiol is necessary for sexually dimorphic expression of AVP in avian and rodent models (Panzica et al., 1998; Plumari et al., 2002). Gene knockout studies have also demonstrated that estradiol regulates both vasopressin and oxytocin specifically through ERα in limbic regions (Choleris et al., 2003; Scordalakes and Rissman, 2004) and through ERβ in hypothalamic areas (Nomura et al., 2002; Shughrue et al., 2002).

The effects of estrogen on the avian song system are well studied and ERα is expressed in or near several vocal control centers. Neural estrogen production is known to contribute to seasonal plasticity of vocal brain nuclei volume and appears to play a role in the generation of sexual dimorphisms during development (for review, see Ball et al., 2002). While similar mechanisms may contribute to sexual dimorphisms in the vocal motor system in midshipman fish and other vocal vertebrate groups in general, the rapid effects of steroids on vocal motor patterning have only just been demonstrated in midshipman fish; 17β-estradiol, 11-ketotestosterone, and glucocorticoids all modulate the rhythmic output of the vocal pattern generator in the hindbrain – spinal cord (Remage-Healey and Bass, 2004). However, unlike other steroids, modulation by 17β-estradiol is short-lived (up to 30 minutes) and restricted to the hindbrain – spinal cord (the other steroids have effects at midbrain levels as well that can last for ≥60 minutes). Specificity of this response was shown, as 17α-estradiol is ineffective at modulating vocal activity. These effects support the hypothesis that high levels of aromatase

Fig. 11. Distribution of ERα and aromatase in the peripheral auditory system of females. Functional anatomy of the inner ear revealed by HCS-1 (hair cell specific) antibody (brown reaction product, Nissl counterstain, A–D). **A:** Sagittal section through the saccular epithelium shows the hair cell layer (HC) relative to ganglion cells (GC) that are positioned within the saccular branch of the eighth nerve that innervates the HC. **B:** High magnification of A showing hair cell morphology; a hair cell nucleus is also indicated (arrow). **C,D:** Oblique section through the saccular epithelium showing variable levels through the hair cell layer (HC). Top arrow in C points to basal hair cell layer, bottom arrow points to tips of hair cells. D is high magnification of C, arrowhead indicates same ganglion cell in both photos. **E:** In situ hybridization for ERα in adjacent section to D demonstrates ERα mRNA (arrows) in branches of the auditory nerve just outside the hair cell layer. **F:** Darkfield visualization of ERα mRNA in a horizontal section through nerve branches just beneath the hair cell layer shows clusters of hybridization signal. **G:** Teleost-specific aromatase antibody reveals round and fusiform aromatase-positive cells and processes (brown reaction product) in the eighth nerve proximal to the saccular epithelium. **H:** Double-label immunofluorescence using aromatase (green) and neuronal (soma) specific Hu (red) antibodies reveals aromatase in ganglion cell somata (bright yellow) and their processes (green) in the eighth nerve (near level of G). H, inset: high magnification of aromatase expression in bipolar ganglion cells (yellow) and processes (green) (center and upper left). Scale bars = 150 μm in A; 30 μm for B; 100 μm in C; 50 μm for D,E; 100 μm in F; 50 μm in G (applies to G,H inset); 100 μm for H.

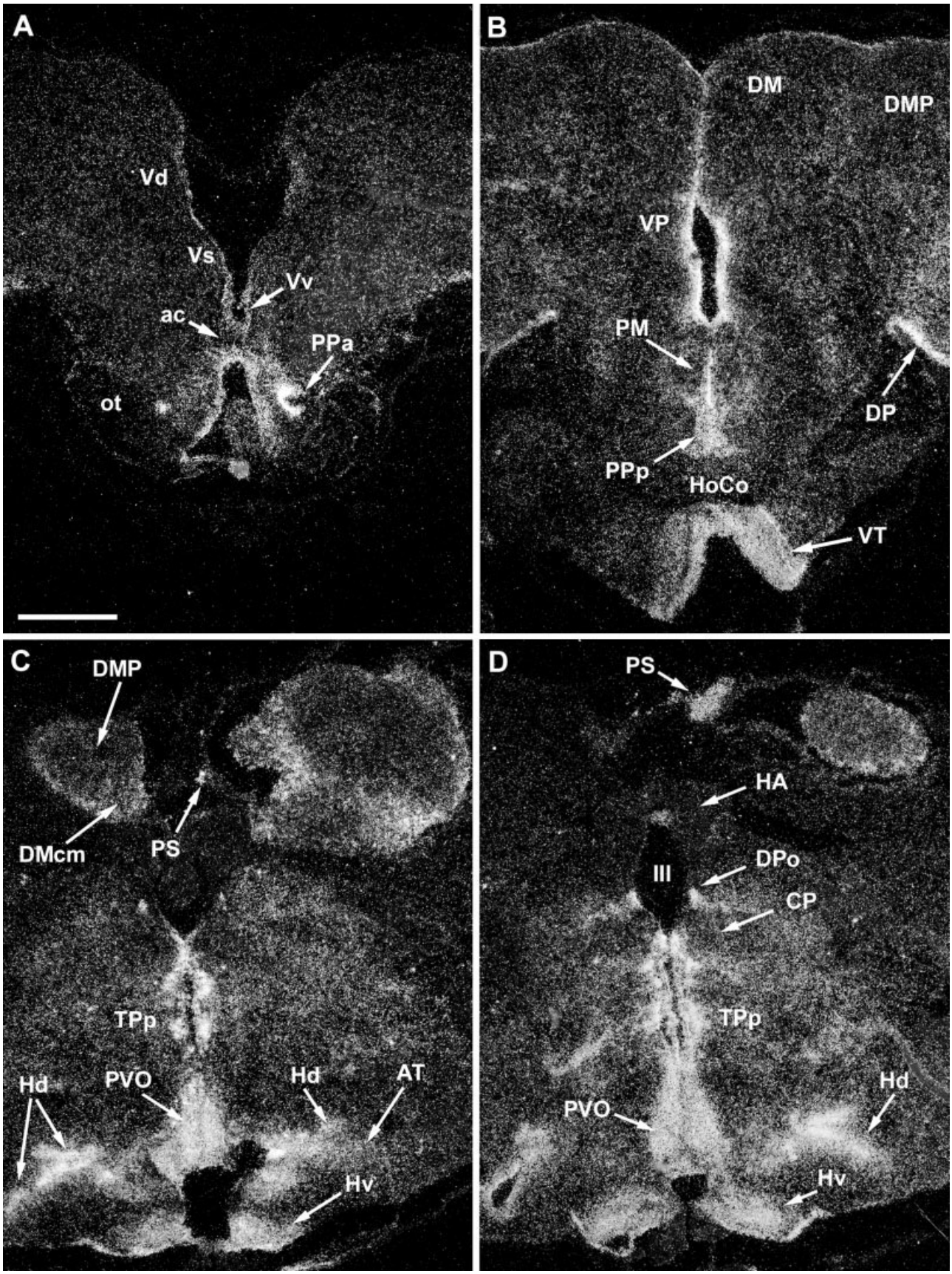
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Figure 12

TABLE 1. Comparative Neuroanatomy of ERα mRNA Distribution

Brain area	Midshipman (ERα mRNA)	Other teleosts (ERα mRNA)[1]	Other teleosts ([3H] estradiol)[2]	Proposed mammalian homolog (ERα mRNA)[3]
Ventral telencephalon				
Olfactory bulb (OB)	+	NR	−	−
Ventral nucleus (Vv)	+ (olf)	(+)[a] (protein only)	+	+ Lateral septal nucleus[4]
Supracommissural nucleus (Vs)	+ (olf)	NR	+	+ Bed nucleus of stria terminalis (BST)/Basal amygdala[4]
Postcommissural nucleus (VP)	+ (olf)	NR	(+)*	+ Basal amygdala[4]
Dorsal telencephalon				
Central zone (DC)/Central medial division of medial zone (DMcm)	+ (olf)	NR	−	+ Pallial amygdala[5]
Posterior nucleus of medial zone (DMP)	+	NR	−	+ Pallial amygdala[5]
Dorsal posterior zone (DP)	+ (olf)	NR	−	+ piriform cortex[5]
Preoptic area				
Anterior parvocellular (PPa)	+ (fVAC)	+	+	+ Anteroventral periventricular (AVPV)[6]
Magnocellular (PM)	+	NR	+	+ Medial (MPN)[6]
Ventral hypothalamus				
Anterior tuberal (AT)	+ (fVAC)	NR	(+)*	+ Ventromedial hypothalamus (VMH)[6]
Periventricular (Hv/Hd)	+	+	+	+ Arcuate/periventricular (PVa, PVp)[6]
Thalamus				
Periventricular nucleus of posterior tuberal (TPp)	+	NR	(+)*	?
Pineal	+	(+) (in vitro only)[b]	NR	(+) Pineal (human, protein)[c]
Brainstem				
Periacqueductal gray (PAG)	+ (mVAC)	NR	−	+ PAG
Optic tectum	+	NR	(+)*	+ Superior colliculus
Torus semicircularis	+ (A)	NR	(+)*	− Inferior colliculus
Sonic motor nucleus	+ (V)	NR	−	− Hypoglossal[7]
Peripheral tissues				
Sensory epithelium (inner ear)	+ (A)	NR	NR	+ Inner ear (protein only)[d]

[1]Atlantic croaker (Hawkins et al., 2000), rainbow trout (Menuet et al., 2001), zebrafish (Menuet et al., 2002), eelpout (Andreassen et al., 2003), [a]rainbow trout (Anglade et al., 1994); [b]rainbow trout (Begay et al., 1994).
[2]Oyster toadfish, paradise fish, goldfish, platyfish (for summary see Fine et al., 1990).
( )* toadfish only.
[3]Simerly et al., 1990, Shughrue et al., 1997, Laflamme et al., 1998, Kruijver et al., 2002; [c]Luboshitzsky et al., 1997, [d]Stenberg et al., 1999, 2001.
[4]Based on Northcutt, 1995.
[5]Based on Braford, 1995.
[6]Based on location and/or function and presence of ERα.
[7]Bass and Baker, 1997.
A, auditory; fVAC, part of forebrain vocal-acoustic complex; mVAC, part of midbrain vocal-acoustic complex; olf, receives olfactory projections; V, vocal; NR, not reported; +, present; −, absent.

in and around the vocal motor nucleus (Forlano et al., 2001; Schlinger et al., 1999) along with ERα expression in the SMN (this report) provide the basis for local estrogen-dependent modulation of the hindbrain – spinal vocal pattern generator. The potential for estradiol regulation of catecholaminergic inputs to the SMN (see earlier discussion) provides yet another level of influence for estrogen on the activity of the hindbrain – spinal vocal circuitry.

Fig. 12. Darkfield visualization of aromatase mRNA distribution in the forebrain. **A:** Section through the anterior parvocellular preoptic area, PPa, (adjacent to Fig. 4B) showing broad distribution of aromatase throughout the telencephalon but concentrated in the PPa and along all ventricular surfaces. **B:** Section through the caudal preoptic area near level shown in Figure 5A. Aromatase expression is ubiquitous, but highest along the ventricular midline, in the medial magnocellular preoptic area (PM), posterior parvocellular preoptic area (PPp), ventrolateral DP (posterior dorsal telencephalon), and the ventral tuberal hypothalamus (VT, part of the descending vocal-acoustic pathway; see Goodson and Bass, 2002). **C,D:** Adjacent sections to Figure 7A,B (prespawning, recrudescing females) showing intense hybridization signal along the midline third ventricle (III), periventricular posterior tuberal nucleus (TPp) and paraventricular organ (PVO), periventricular hypothalamic areas (Hd, Hv), anterior tuberal nucleus (AT), dorsal and central posterior thalamus (DPo, CP), and most caudal telencephalon in DMP and DMcm (posterior and central medial divisions of the medial zone of area dorsalis). Scale bar = 500 μm.

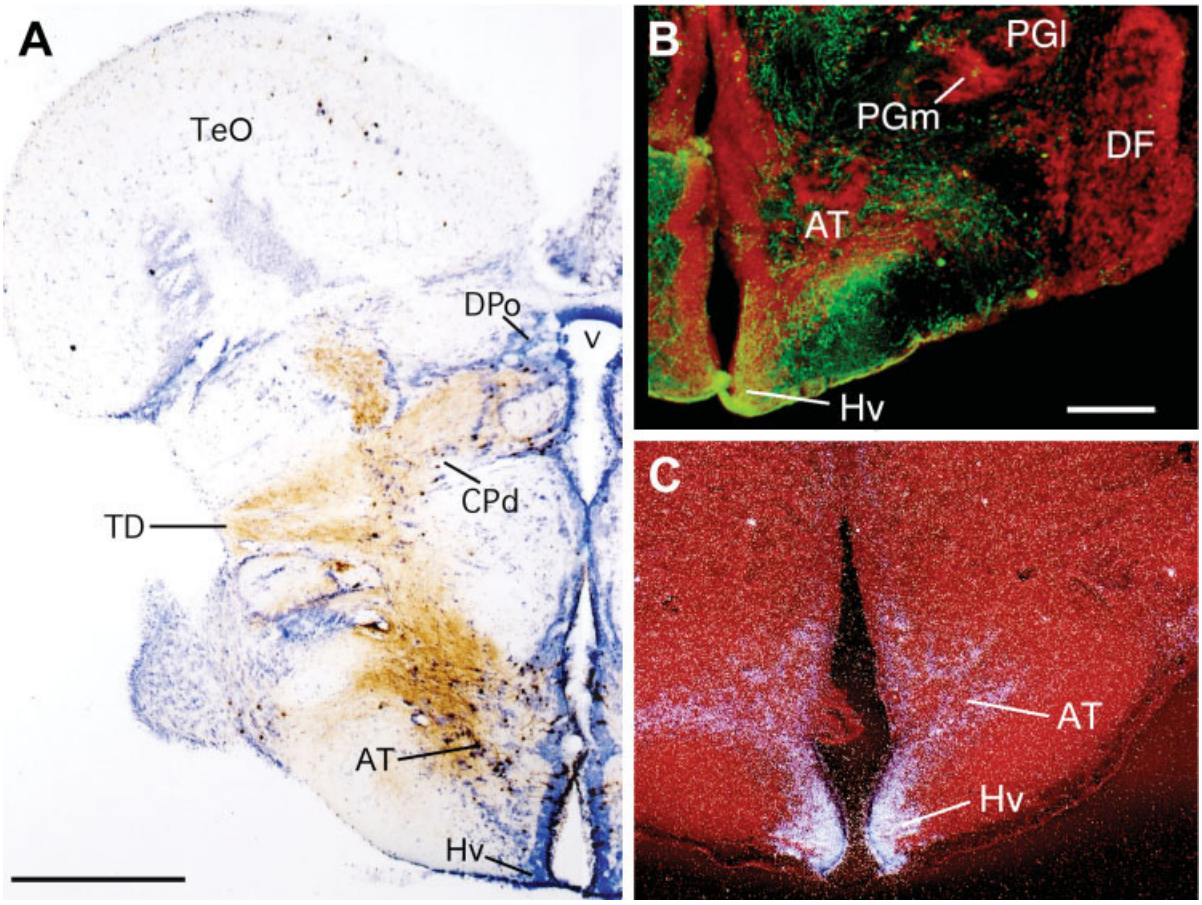

Fig. 13. Vocal-acoustic-neuropeptide-steroid hormone integration center at the anterior tuberal nucleus (AT) of the hypothalamus in the plainfin midshipman fish. **A:** Low-power montage showing labeled cells and fibers in AT after a neurobiotin injection into the ventral tuberal nucleus (VT, see Fig. 12B for location) which indicates connectivity (brown reaction product) between both vocally active areas. Also note projections into the diffuse portion of the central posterior (CPd) nucleus, a part of the auditory thalamus (modified from Goodson and Bass, 2002). **B:** Distribution of mesotocin- and oxytocin-like immunoreactivity (green) in AT. Red is a fluorescent Nissl-stain. Vasotocin is also found in this same area (modified from Goodson et al., 2003). **C:** Darkfield visualization of ERα mRNA in AT at approximately the same level shown in B. Scale bars = 500 μm in A; 200 μm in B (applies to B,C).

The rapid action of 17β-estradiol on the sonic motor system suggests the presence of a membrane-bound estrogen receptor (Remage-Healey and Bass, 2004) similar to that proposed for rapid signaling in guinea pig hypothalamic neurons (Qiu et al., 2003). Estrogen receptor α has been documented in the plasma membrane of neurons, including dendritic spines, axon terminals, and astroglial processes in the hippocampus and hypothalamus (for reviews, see Beyer et al., 2003; McEwen, 2002; Toran-Allerand, 2004). Recently, Kruijver et al. (2002) demonstrated that virtually all areas which showed nuclear staining also showed cytoplasmic staining of ERα in the human hypothalamus. Thus, a teleost-specific ERα antibody should help elucidate cytoplasmic or nuclear localization of ERα corresponding to mRNA expression in the midshipman brain. Furthermore, recent studies by Balthazart et al. (2001) demonstrate rapid changes in brain aromatase activity (within 5 minutes), which suggests nongenomic effects of estrogens may be facilitated by rapid regulation of estrogen production. Therefore, rapid effects of estradiol on the vocal motor system seen experimentally (Remage-Healey and Bass, 2004) may occur naturally by rapid changes in the availability of locally formed estradiol via rapid changes in aromatase activity within the sonic motor nucleus. The anatomical distribution of aromatase within and around the SMN is perfectly localized for this mechanism to occur in midshipman fish.

Peripheral auditory system

Underwater playback experiments show that the male's advertisement call or "hum" is both necessary and sufficient for reproductive females to detect and locate courting males (Bass and McKibben, 2003). Furthermore, the female sacculus (main auditory endorgan of the inner ear) undergoes a dramatic increase in the degree of temporal encoding of the higher frequency harmonics of the male's hum when sampled during the reproductive season compared to the nonreproductive season. This seasonal plasticity in auditory encoding likely allows females to better detect and locate a mate (Sisneros and Bass, 2003). The natural seasonal change in female auditory sensitivity can be mimicked by implanting nonreproductive females with

either 1) testosterone (T) or 2) 17β-estradiol. In contrast to the rapid effects of estradiol on vocal motor physiology, these changes occur over several weeks, suggesting a long-term, genomic activation of estradiol on the physiology of the peripheral auditory system (Sisneros et al., 2004b). The time course of action corresponds to a seasonal peak of circulating steroids (T and estradiol) in females ~1 month before the summer spawning season (Sisneros et al., 2004a). Thus, these findings prompted the investigation of ERα in the peripheral auditory system of midshipman fish. Since both T and estradiol have identical effects on the auditory system, it is likely the change in sensitivity may be due entirely to estradiol because circulating estradiol is 2–3-fold higher than T, and the action of T may be due to local conversion to estradiol by aromatase which is found at high levels in the midshipman brain (Forlano et al., 2001; Schlinger et al., 1999) and auditory nerve (this report).

Although ERα immunoreactivity (not mRNA) was described in the cochlea of humans and rodents, its functional significance remains unknown (Stenberg et al., 1999, 2001). This study reveals ERα mRNA for the first time in the inner ear of any vertebrate and it appears to be localized in nerve branches proximal to the hair cell layer in the sensory epithelium. Thus, ERα may modulate hair cell properties postsynaptically through mechanisms such as synaptogenesis (McEwen et al., 2001), by changing filtering properties at the hair cell-afferent synapse (Koppl, 1997; Palmer and Russell, 1986; Weiss and Rose, 1988) or by inducing proliferation of new hair cells. Localization of aromatase-immunoreactive ganglion cells in the auditory branch of the eighth nerve supports the hypothesis of an entirely estradiol-dependent effect of modulation by local production of estradiol.

CONCLUSIONS

This study supports the hypothesis that estrogens, produced in either the gonad (females) or the central and peripheral nervous systems (males and females), of teleosts likely serves multiple functions, not only to regulate the neuroendocrine basis of reproduction, but also to serve as a fast-acting modulator of neural activity via either membrane or cytoplasmic receptors (e.g., vocal motor system), and/or to govern changes through classic ligand-activated gene expression (e.g., peripheral auditory system). Moreover, all or some of these mechanisms likely involve estrogenic interactions with other neurochemicals (e.g., neuropeptides and catecholamines) that together act locally (e.g., in AT, the vocal-acoustic hypothalamus) to influence the neural substrates of naturally selected behaviors (e.g., vocalizations). This study exemplifies the conserved nature of ERα mRNA expression in discrete brain areas across vertebrate groups as well as novel functional significance in the plasticity of the vocal and auditory systems. These results also show for teleost fish that the distribution and relative abundance of ERα mRNA expression contrasts with the wider distribution and greater abundance of aromatase mRNA and protein (e.g., in the telencephalon and sonic motor nucleus), suggesting that other genomic and nongenomic mechanisms are mediating the effects of estrogens and neuroestrogens on motor and sensory circuits in the adult vertebrate brain.

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