Distribution of Sex Steroid Hormone Receptors in the Brain of an African Cichlid Fish, *Astatotilapia burtoni*

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ABSTRACT

Sex steroid hormones released from the gonads play an important role in mediating social behavior across all vertebrates. Many effects of these gonadal hormones are mediated by nuclear steroid hormone receptors, which are crucial for integration in the brain of external (e.g., social) signals with internal physiological cues to produce an appropriate behavioral output. The African cichlid fish *Astatotilapia burtoni* presents an attractive model system for the study of how internal cues and external social signals are integrated in the brain as males display robust plasticity in the form of two distinct, yet reversible, behavioral and physiological phenotypes depending on the social environment. In order to better understand where sex steroid hormones act to regulate social behavior in this species, we have determined the distribution of the androgen receptor, estrogen receptor alpha, estrogen receptor beta, and progesterone receptor mRNA and protein throughout the telencephalon and diencephalon and some mesencephalic structures of *A. burtoni*. All steroid hormone receptors were found in key brain regions known to modulate social behavior in other vertebrates including the proposed teleost homologs of the mammalian amygdalar complex, hippocampus, striatum, preoptic area, anterior hypothalamus, ventromedial hypothalamus, and ventral tegmental area. Overall, there is high concordance of mRNA and protein labeling. Our results significantly extend our understanding of sex steroid pathways in the cichlid brain and support the important role of nuclear sex steroid hormone receptors in modulating social behaviors in teleosts and across vertebrates. J. Comp. Neurol. 518:3302–3326, 2010.

INDEXING TERMS: estrogen receptor; androgen receptor; progesterone receptor; hypothalamus; social behavior network; mesolimbic reward system

Sex steroid hormones—androgens, estrogens, and progestins—are ubiquitous in all vertebrates and modulate a variety of neural processes and behavior such as reproduction, aggression, learning, and memory (Hull and Dominguez, 2007; Westberg and Eriksson, 2008; Luine, 2008; Galea et al., 2008). Each of these sex steroid hormones modulate these processes through specific receptors in dedicated neural circuits that are well characterized in many species of birds and mammals (Ball and Balthazart, 2004; Dulac and Kimchi 2007). Within these neural circuits, gonadal hormones can have both organizational and activational effects. Classically, steroid hormones organize the brain during development and then activate the structures organized in development later in life (Phoenix et al., 1959; Young et al., 1964; McEwen, 1980). However, the relative extent to which organizational or activational effects shape an organism's brain and behavior can vary considerably across species. This is well illustrated by considering two types of polymorphic systems: one in which the male phenotypes are fixed throughout their adult life and another in which males switch between reproductive morphs throughout the adult lifespan (Knapp, 2004). Organizational effects appear strongest in fixed phenotypic systems, whereas activational effects of steroid hormones and their receptors are more substantial in plastic phenotypic systems (Moore, 1991; Thompson and Moore, 1992). Thus, to understand the role of steroid hormones and their receptors in actively modulating complex behavior, a model

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system that is behaviorally plastic is advantageous. Here we characterize the distribution of androgen receptors, estrogen receptors, and the progesterone receptor in the brain of the African cichlid fish, *Astatotilapia burtoni*, which has become an established model system for the study of hormonal and environmental modulation of phenotypic and neural plasticity (Hofmann, 2003; Robinson et al., 2008).

Stimuli in the (social) environment are transduced by sensory systems and can have both immediate and long-term effects on brain processes and behavior. The modulation of gene expression levels constitutes one key channel for affecting such long-term changes (Aubin-Horth and Renn, 2009). In addition to internal physiological cues, production of social behavior requires an integration of external cues in the brain where signals are processed and behavioral decisions implemented by dedicated brain circuits. Steroid hormones are excellent candidates for integrating environmental inputs into gene expression changes, which then in turn can alter neural circuit function and properties (Ball and Balthazart, 2004). Changes in external signals, such as altering day length or social environment, can influence the effects of hormones on behavior (Trainor et al., 2007; Goymann, 2009). However, gonadal hormones can also have rapid nongenomic effects that can play an important role in behavior (Remage-Healey and Bass, 2006; Mani et al., 2009).

Each gonadal hormone is associated with one or more conserved nuclear receptor that mediates enduring effects of the hormone by influencing gene transcription. Upon ligand binding, receptors dimerize and act cooperatively with coactivators and other DNA-binding proteins to interact with regulatory sequences in promoter regions called hormone response elements (Tetel, 2009). The specificity of action of steroid hormone receptors is due to specificity of the receptor to both its ligand and its DNA response element as well as the spatial and temporal expression of the steroid hormone receptor itself. The distribution of androgen and estrogen receptors in the brain has been studied in several teleost species including goldfish, the oyster toadfish, zebrafish, midshipman, sea bass and Atlantic croaker (androgen receptor: Fine et al., 1996; Gelinas and Callard, 1997; Forlano et al., 2009; estrogen receptors: Menuet et al., 2002; Hawkins et al., 2005; Forlano et al., 2005; Muriach et al., 2008). The neural distribution of the progesterone receptor has been studied in zebrafish, although only a few brain regions are described (Hanna et al., 2010). Surprisingly, little is known about the distribution of these receptors in the brains of teleosts with plastic behavioral phenotypes, even though this information would give us a better understanding of which brain regions may be sites of modulation of neural and behavioral plasticity by gonadal hormones.

Abbreviations

An	Anterior thalamic nucleus
AC	Anterior commissure
aTn	Anterior tuberal nucleus
Cn	Central nucleus of the inferior lobe
CP	Central posterior thalamic nucleus
D	Dorsal (pallial) part of the telencephalon
Dc	Central part of D
Dc-2	Subdivision of Dc
Dd	Dorsal part of D
DH	Dorsal hypothalamus
Dl	Lateral part of D
Dld	Dorsal part of Dl
Dlg	Granular part of Dl
Dlv	Ventral part of Dl
Dlvv	Ventral part of Dlv
Dm	Medial partof D
Dm-1,2,3	Subdivisions of Dm
Dm2c	Caudal part of Dm-2
Dn	Diffuse nucleus of the inferior lobe
Dp	Posterior part of D
Dx	Unassigned part of D
E	Entopeduncular nucleus
Gn	Glomerular nucleus
H	Habenula
HC	Horizontal commissure
IL	Inferior lobe
LHn	Lateral hypothalamic nucleus
lPGn	Lateral preglomerular nucleus
LR	Lateral recess
LT	Longitudinal torus
LZ	Limited zone of the diencephalon
MB	Mammillary body
mPGn	Medial preglomerular nucleus
nLT	Nucleus of the lateral torus
OB	Olfactory bulb
OPT	Optic tract
OT	Optic tectum
P	Pituitary
PAG	Periaqueductal gray
PGCn	Preglomerular commissural nucleus
PN	Prethalamic nucleus
POA	Preoptic area
PPd	Dorsal periventricular pretectal nucleus
PPr	Rostral periventricular pretectal nucleus
pTGN	Preglomerular tertiary gustatory nucleus
PVO	Paraventricular organ
ST	Semicircular torus
TPp	Periventricular nucleus of the posterior tuberculum
V	Ventral (subpallial) division of the telencephalon
Vc	Central part of V
Vd	Dorsal nucleus of V
Vdc	Caudal part of Vd
Vdr	Rostral part of Vd
VH	Ventral hypothalamus
Vi	Intermediate part of V
Vl	Lateral part of V
VM	Ventromedial thalamic nucleus
Vp	Postcommissural nucleus of V
vPPn	Ventral portion of the periventricular pretectal nucleus
Vs	Supracommissural nucleus of V
Vsl	Lateral part of Vs
Vsm	Medial part of Vs
vTn	Ventral tuberal nucleus
Vv	Ventral part of V

The African cichlid, *A. burtoni*, is an excellent model system to study the mechanisms by which gonadal hormones modulate neural and behavioral plasticity. In this species, males display robust phenotypic plasticity in the form of two distinct phenotypes. Dominant males are colorful, reproductively active, and defend territories where they court and spawn with females. Subordinate males have cryptic coloration, are reproductively suppressed, and school with females. This species presents an attractive model system for the study of activational effects of hormones and their receptors as individuals are "plastic" and change from one phenotype to another depending on the immediate social environment (Hofmann and Fernald, 2001). The social behavior of these fish is complex, yet stereotyped and easily quantified, adding to their potential as a model system (Fernald and Hirata, 1977a,b). Additionally, in *A. burtoni* we can investigate the interaction between an individual and its social environment, as both social and hormonal status can exert profound influences on neural circuitry and behavior (Hofmann, 2003).

The influence of social status and behavioral plasticity on steroid hormone levels, especially androgens, has been studied extensively in *A. burtoni*. Dominant males have significantly higher circulating levels of both androgens (testosterone and 11-ketotestosterone) and estradiol than subordinate males (Parikh et al., 2006a; Greenwood et al., 2008; Munchrath and Hofmann, in prep.). Fernald (1976) showed that exogenous testosterone increased aggressive displays in dominant males, although it remains unclear whether this effect was mediated by androgen receptors or by estrogen receptors via aromatase. There are also physiological consequences to a change in social status, as after social defeat androgen levels will decrease in dominant males within 24 hours (Parikh et al., 2006b). Importantly, the expression of androgen and estrogen receptors varies within these social phenotypes. Burmeister et al. (2007) measured the levels of androgen and estrogen receptor mRNA in gross brain dissections in *A. burtoni* and found that in the anterior portion of the brain (which comprised the entire telencephalon and a portion of the POA), dominant males had higher levels of ARα, ARβ, ERβa, and ERβb mRNA compared with subordinate males. Yet despite the potentially important role of steroid hormones in modulating behavioral plasticity and social dominance in this species, knowledge of the detailed distribution of sex steroid hormone receptors in the brain is lacking.

Based on insights in mammals, birds, and teleosts, there are two neural networks that seem to regulate social behavior and/or encode the salience of (social) stimuli. Many studies indicate that the "reward system" (including but not limited to the midbrain dopaminergic system) is the neural network where evaluations of stimulus salience are made (Deco and Rolls, 2005; Wickens et al., 2007). The neural substrate of social behaviors has been described by Newman (1999) as "social behavior network" in mammals, and has been expanded to reptiles, birds, and teleosts (Newman, 1999; Crews, 2003; Goodson, 2005). The core nodes of Newman's network are involved in multiple forms of social behavior, are reciprocally connected, and—by definition—contain sex steroid hormone receptors. Although the brain regions involved in the dopaminergic reward system and Newman's social behavior network are well studied in mammals, homologizing these brain areas with structures in the teleost brain has been controversial (Nieuwenhuys et al., 1998; Northcutt, 2008). However, a consensus is emerging from developmental, neurochemical, hodological, and lesion studies that provides support for at least partial homologies for most of the relevant areas in the teleost brain (Rink and Wullimann, 2001, 2002; Portavella et al., 2002; Wullimann and Mueller, 2004; Northcutt, 2006, 2008; Bruce and Braford, 2009).

The main aim of this study was to test the hypothesis that the androgen receptor (AR), estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and the progesterone receptor (PR) are widely distributed throughout fore- and midbrain of a teleost with plastic behavioral phenotypes. We also predicted that these steroid receptors are expressed in brain regions important for the regulation of social behavior and evaluation of stimulus salience in the African cichlid fish *A. burtoni*.

MATERIALS AND METHODS

Animals

Astatotilapia burtoni from a wild-caught stock population were kept in aquaria under conditions mimicking their natural environment (Fernald and Hirata, 1977b): pH 8.0, 28°C water temperature, and 12h:12h light:dark cycle with 10 minutes each dusk and dawn periods. Gravel substrate and terracotta shelters provided the substrate that facilitates the establishment and maintenance of territories necessary for reproduction (Fernald and Hirata, 1977a). Fish were fed every day with cichlid flakes (Arcata Pet Supplies). The animals chosen for this study were dominant and subordinate males as described by Fernald (1976), who had been in their respective social states for at least 4 weeks. Dominant males were identified as aggressively defending a territory within the tank, courting females, and displaying bright color and an eye bar. Subordinate males were identified by absence of a territory, schooling with the females, fleeing from territorial males, and lack of bright body coloration and eye bar. All work was carried out in compliance with the

Institutional Animal Care and Use Committee at the University of Texas at Austin.

Where possible the neuroanatomical nomenclature of Fernald and Shelton (1985) for the diencephalon and mesencephalon and Burmeister et al. (2009) for the telencephalon was followed. However, for the prethalamic nucleus (PN), we adopted the nomenclature of Meader (1934), although this region has been identified as the anterior preglomerular nucleus (aPGn) in Fernald and Shelton (1985). This was done to avoid confusion of functionally different regions in percomorphs and cyprinids (Braford and Northcutt, 1983; Yamamoto and Ito, 2005, 2008; Northcutt, 2006). For the central nucleus of the inferior lobe we followed the nomenclature according to Ahrens and Wullimann (2002) and Yang et al. (2007). The nomenclature for the periaqueductal gray was used according to Forlano et al. (2001). Parvocellular, magnocellular, and gigantocellular portions of the preoptic area were identified as described in Braford and Northcutt (1983) based on cell size and location.

Cloning of the A. burtoni PR full cDNA

With the exception of the PR, sequences for all *A. burtoni* sex steroid hormone receptors were available in GenBank (see below). In order to obtain the *A. burtoni* PR sequence, we designed primers against the Nile tilapia, *Oreochromis niloticus*, PR mRNA (GenBank accession number AB110982) and cloned a 359-bp fragment of the *A. burtoni* PR from whole brain cDNA. (See Table 1 for information on primer sequences.) The remainder of the full-length sequence corresponding to the 5′ and 3′ end of the coding region and the 5′ and 3′ untranslated region was cloned by RACE (ClonTech, Palo Alto, CA) according to the manufacturer's instructions. The entire sequence has been submitted in GenBank (accession number FJ605735).

TABLE 1.

Primer Information for Cloning the Progesterone Receptor

PR cloning reaction	Primer sequence
PR cloning, forward primer	5′-GCACCTGATCTGATTCTTAGCCAG
PR cloning, reverse primer	5′-GCCTGGATGAAGGTACTCAAACAG
Outer 5′-RACE	5′-GGCATCCATGAGTTTGGTGAGGTGG
Nested 5′-RACE	5′-TTTGAATAGCCTTGGTCAGCTCCCG
Outer 3′-RACE	5′-CAAATGAAGGAGAAGGGCATCGTGG
Nested 3′-RACE	5′-CCCAGCGATTCTACCACCTCACCAAAC

In situ hybridization (ISH)

Dominant (n = 8) and subordinate (n = 8) males were sacrificed and their brains rapidly dissected, fresh frozen in O.C.T. (Optimal Cutting Temperature Compound, Tissue-Tek, Torrance, CA) on a block of dry ice and stored at −80°C. Brains were then sectioned on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific, Portsmouth, NH) in six series that were stored at −80°C for at least 6 weeks until processing for ISH. Each of the six steroid hormone receptors was analyzed in each individual. Sections were fixed in cold 4% paraformaldehyde (pH 7.2) for 10 minutes and washed in 1× phosphate-buffered saline (PBS; pH 7.4). The sections were then incubated in 0.1 M triethanolamine (TEA) (pH 8.0) for 10 minutes followed by 15 minutes in freshly prepared TEA/0.25% acetic anhydride, rinsed in 2× SSC, dehydrated in increasing ethanol series, air-dried, and stored at −80°C. Riboprobes were reverse-transcribed in the presence of fluorescein-labeled UTP (Roche, Indianapolis, IN) using a T7/SP6 Maxiscript in vitro transcription kit (Ambion, Austin, TX) to produce antisense or sense fluorescein-labeled probes. All probes were designed to include the ligand-binding domain of each receptor. (See Table 2 for primer information.) The template used to make the ARα probe was 646 bp in length (GenBank accession number: AF121257); the ARβ probe was 516 bp in length (GenBank accession number: AY082342); the ERα probe was 788 bp in length (GenBank accession number: AY422089); the ERβa probe was 670 bp in length (GenBank accession number: DQ862128); the ERβb probe was 665 bp in length (GenBank accession number: DQ862129); the PR probe was 359 bp in length (GenBank accession FJ605735). Slides were then warmed to room temperature, air-dried, and preequilibrated in hybridization buffer (50% formamide, 5× SSC, 5× Denhardt's solution, 125 mg/mL Baker's yeast tRNA, 250 mg/mL denatured herring sperm DNA) for 2 hours at 65°C. Sections were then incubated in

TABLE 2.

Primer Information for In Situ Hybridization Probe Template

Gene	Forward primer	Reverse primer
Androgen receptor alpha	5′-AATGTGTTTATGAACCCCACGC	5′-TCTTCCGCCTCAGTTTGTCG
Androgen receptor beta	5′-CGGCGGCTTTTCATCACC	5′-AGTTGGAGTTGGGATAAGG
Estrogen receptor alpha	5′-GAAGATGAACCAACCCTAC	5′-TGCCTTGAGGTCCTGAAC
Estrogen receptor beta-a	5′-GCCAAGAAGATTCCAGGGTTTG	5′-TCCAGGTATTTGAAGGTCCGC
Estrogen receptor beta-b	5′-AACCCAGAGTTCATTTCCC	5′-TACACCAGCACCGCATTCTTCC
Progesterone receptor	5′-GCACCTGATCTGATTCTTAGCCAG	5′-GCCTGGATGAAGGTACTCAAACAG

TABLE 3.
Antibody Details

1°	Antigen	Supplier	Source	IHC dilution	Type
PR	Chicken PR purified from oviduct	Abcam (ab2767)	Mouse	1:500	Monoclonal
AR	Human AR: MEVQLGLGRVYPRPPSKTYRGC	Millipore (06-680)	Rabbit	1:250	Polyclonal
ERα	Rat ERα: TYYIPPEAEGFPNTI	Millipore (06-935)	Rabbit	1:1,000	Polyclonal
ERβ	Human ERβ: CSPAEDSKSKEGSQNPQSQ	Invitrogen (51-7700)	Rabbit	1:250	Polyclonal

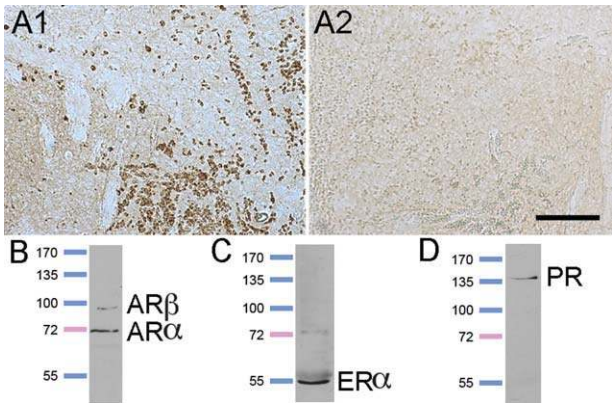

Figure 1. Confirmation of antibody specificity. **A**: Immunohistochemical staining with an ERβ antibody shows distinct cell labeling **(A1)** and this staining is completely blocked when the antibody is preabsorbed with the original antigen prior to immunohistochemical procedure **(A2)**. Western blot was used to confirm specificity of the AR **(B)**, ERα **(C)**, and PR **(D)** antibodies against *A. burtoni* whole brain extract. Scale bar = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

riboprobe overnight at the same temperature. Experimental slides were exposed to antisense fluorescein-labeled probe, whereas control slides were incubated with sense fluorescein-labeled probe. Additional control slides were treated with RNase before hybridization with antisense probe. After the overnight hybridization, slides were processed for detection of mRNA by nonradioactive, nonfluorescent detection. After RNase A treatment at 37°C, sections were washed in a decreasing series of SSC and equilibrated in 150 mM NaCl/100 mM Tris (pH 7.5) at room temperature before incubation in 1:1,000 anti-fluorescein-alkaline phosphatase Fab fragments (Roche) in 0.5% Tween 20/PBS for 2 hours at room temperature. Sections were then washed in 100 mM Tris (pH 7.5). Chromogenic product was formed using BM Purple (Roche) at room temperature until desired darkness was achieved and was terminated simultaneously for all slides within a gene group. Slides were then washed, dehydrated in an ethanol series ending in xylene, and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA).

Immunohistochemistry (IHC)

Dominant (n = 4) and subordinate (n = 4) males were sacrificed and their brains rapidly dissected and incubated in 4% paraformaldehyde in 1× PBS; pH 7.4 at 4°C overnight. Brains were then washed in 1× PBS and cryoprotected in 30% sucrose in 1× PBS overnight at 4°C before embedding in O.C.T. (Tissue-Tek) and storing at −80°C. Brains were then sectioned on a cryostat at 20 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific) in five series that were stored at −80°C until processing for IHC.

Sections were removed from −80°C and air-dried before being fixed in chilled 4% paraformaldehyde in 1× PBS, pH 7.4, for 10 minutes. Sections were then rinsed in PBS and incubated in 3% hydrogen peroxide in PBS for 20 minutes. After washing in PBS, antigen retrieval was performed by incubating in boiling citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0). After two minutes the boiling citrate buffer was replaced two times and incubated for 5 minutes each, followed by a PBS wash. After blocking for 1 hour in blocking solution (5% normal goat serum and 0.3% Triton X-100 in PBS), sections were incubated in primary antibody (AR 1:250, PR 1:500, ERα 1:1,000, ERβ 1:250, see Table 3 for antibody details) in PBS with 2% normal goat serum and 0.3% Triton X-100 at

Figure 2. Distribution of AR mRNA and protein in the telencephalon. Representative sections of the telencephalon are presented as the first image in each panel with AR protein shown as dots on the right side of the brain and ARα mRNA shown as gray shading on the left and ARβ mRNA as gray shading on the right. The density of dots indicating protein corresponds to the density of cells positive for AR-immunoreactivity. The degree of shading for mRNA corresponds to the density of expression. The micrographs in the top panel show ARα and ARβ mRNA **(A2,A3)** and AR protein **(A4)** in the olfactory bulb. Micrographs in the second panel show labeling of ARα and ARβ mRNA **(B2,B3)** and AR protein **(B4)** in Vv. The third panel shows micrographs of ARα and ARβ mRNA **(C2,C3)** and AR protein **(C4)** in Dc. The fourth panel shows micrographs of ARα and ARβ mRNA **(D2,D3)** and AR protein **(D4)** in Vdc and Vsm. The bottom panel shows AR protein in gigantocellular **(E2)**, magnocellular **(E3)**, and parvocellular **(E4)** cells in the POA. Scale bars = 100 μm except E2–E4 = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

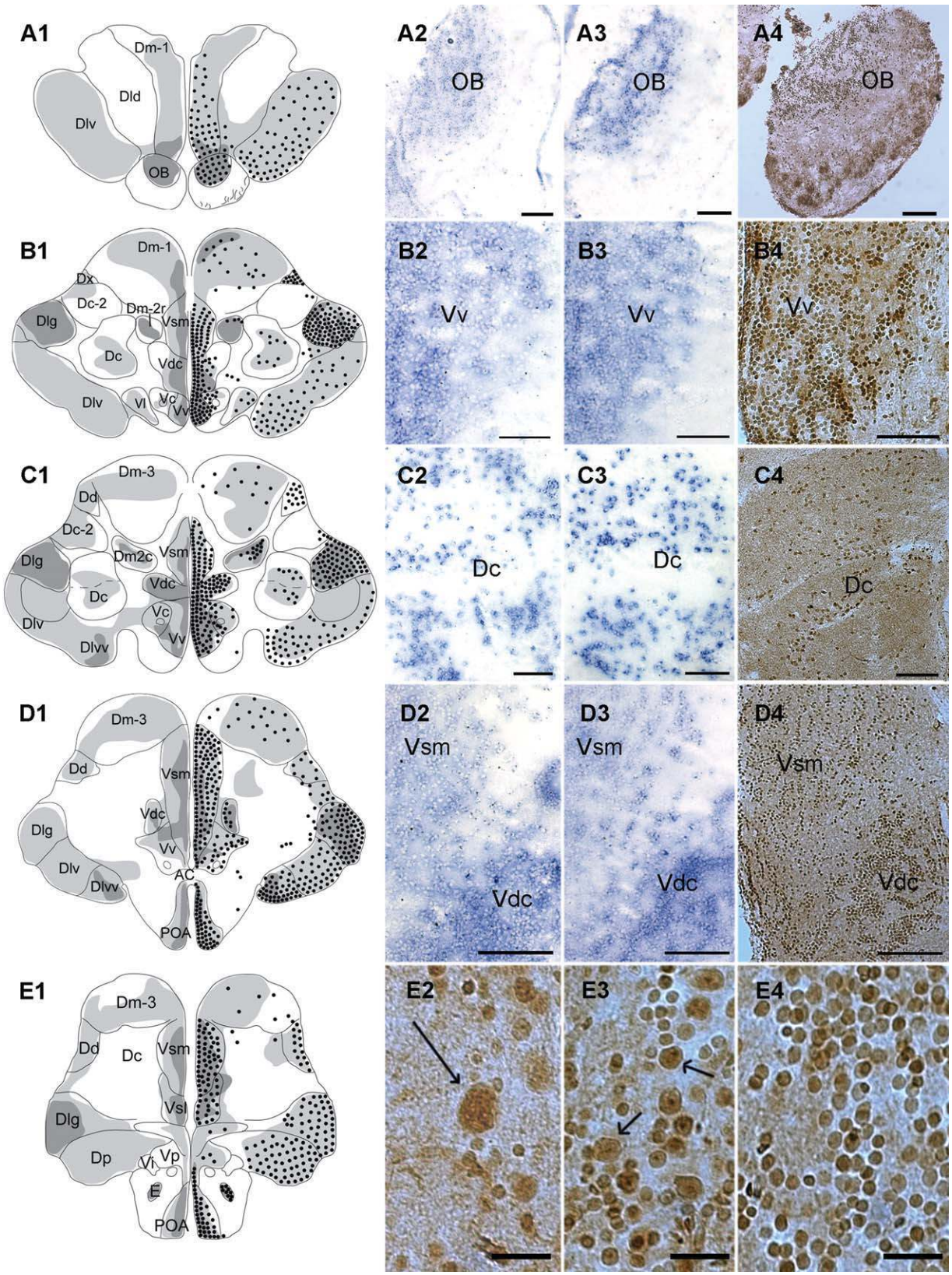
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Figure 2

room temperature overnight. Sections were then rinsed, incubated for 2 hours in a biotinylated goat anti-mouse (PR) or anti-rabbit (AR, ERα, ERβ) secondary antibody (Vector Laboratories, Burlingame, CA), rinsed again, and, after treatment with the ABC peroxidase staining kit (Vector Laboratories) according to the manufacturer's instructions, immunoreactivity was visualized using 3,3′-diaminobenzidine (DAB) substrate (Vector Laboratories). Sections were then dehydrated and coverslipped with Permount (Fisher Scientific, Itasca, IL). For control sections, all procedures were the same except that primary antibody was omitted. All antibodies used in this study were obtained from commercial suppliers, as summarized in Table 3, along with information on antigen, source, and dilution.

Western blot characterization of AR, ERα, and PR antibodies

In order to determine whether these antibodies would bind specifically to the cichlid antigens, we extracted protein from whole brain using a Mammalian Cell Lysis kit (Sigma, St. Louis, MO) according to the manufacturer's instructions. Whole brain protein extract was run on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel in replicate, in which one-half of the gel used for downstream Western blotting and the other half exposed to Coomassie stain to verify protein presence. Whole brain extract on the gel was transferred onto a nitrocellulose membrane overnight. The membrane was then blocked in 5% dry milk in wash buffer (0.5% Triton X-100, 0.1% Tween-20 in 1× Tris-buffered saline [TBS], incubated in primary antibody [1:1,000 AR, 1:5,000 ERα, or 1:2,000 PR in 1× TBS and 2% NaN3]) for 1 hour, washed five times for 3 minutes each in wash buffer, and then incubated in either goat-anti-mouse horseradish peroxidase (HRP)-conjugated antibody (PR) or goat-anti-rabbit HRP-conjugated antibody (AR, ERα; Southern Biotechnology, Birmingham, AL) in blocking solution for 30 minutes. After washing five times for 3 minutes each with wash buffer, the membrane was exposed to HRP substrate (Immobilon Western, Millipore, Bedford, MA) and exposed to film for 2 minutes. Using the AR antibody, two bands were visualized putatively representing ARα and ARβ at the predicted sizes of 90 kD and 78 kD, respectively (Fig. 1B). Using the ERα antibody, one large band was visualized near 55 kD, near the expected size of the teleost ERα (Fig. 1C). The predicted size of the teleost ERβ is near 75 kD. Using the PR antibody, one large band was visualized near 140 kD, the putative size of the teleost PR (Fig. 1D). As can sometimes be the case with immunoblotting, the ERβ antibody did not provide a signal on the Western blot. We found, however, that preabsorption of the ERβ antibody with 10 μg per mL of original antigen for 1 hour at room temperature prior to immunohistochemistry blocked all signal (Fig. 1A). This result does not rule out that the ERβ antiserum might still crossreact with another protein in the brain. However, all antisera used in this study, including the one against ERβ, showed high concordance with mRNA expression patterns as determined by in situ hybridization, indicating specificity.

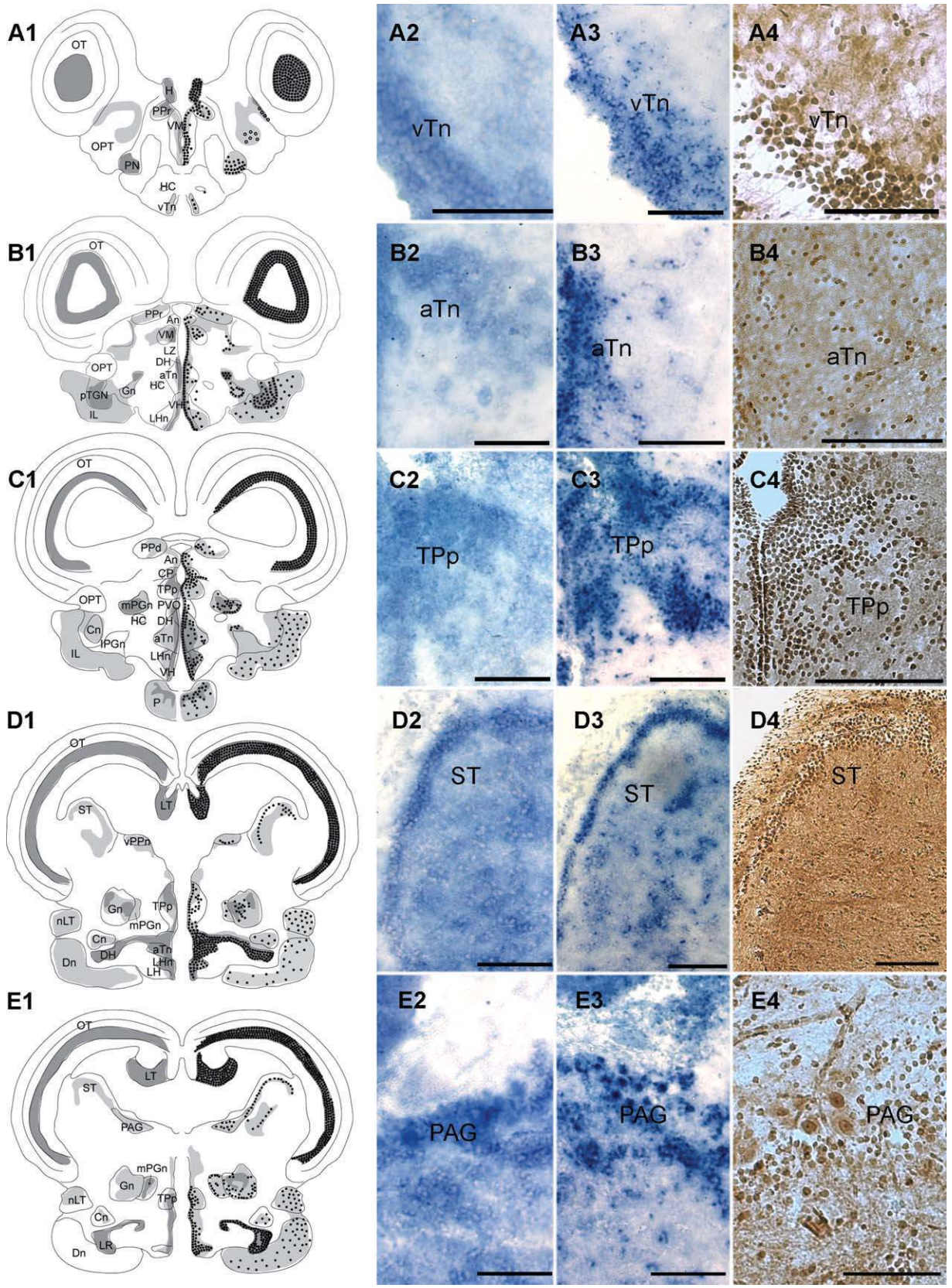
Photomicroscopy

Brightfield optics were used to visualize immunohistochemical staining throughout the brain at low (5×) and high magnification (10×). Photographs were taken with a digital camera (AxioCam MRc, Zeiss) attached to a Zeiss AxioImager.A1 AX10 microscope (Zeiss) using the AxioVision (Zeiss) image acquisition and processing software. Images were compiled and brightness- and contrast-enhanced in Adobe Photoshop CS3 (San Jose, CA).

RESULTS

Sex steroid receptors are widely distributed throughout the male cichlid fore- and midbrain. In the following, we present a distribution map along with representative photomicrographs of representative brain areas for each steroid hormone receptor separately. For each representative section of the map, the nomenclature is displayed on the left side while protein signal as determined by immunohistochemistry is represented by dots on the right side. The density of dots representing protein indicates qualitatively the density of cells positive for the protein of

Figure 3. Distribution of AR mRNA and protein in the diencephalon and mesencephalon. Representative sections of the diencephalon and some mesencephalic structures are depicted in the left column with AR protein represented by dots on the right, ARα mRNA as gray shading on the left, and ARβ mRNA as gray shading on the right. The density of dots indicating protein corresponds to the density of cells positive for AR-immunoreactivity. The degree of shading for mRNA corresponds to the density of expression. Micrographs in the top row show ARα and ARβ mRNA **(A2,A3)** and AR protein **(A4)** in the ventral tuberal nucleus. Micrographs in the second row show ARα and ARβ mRNA **(B2,B3)** and AR protein **(B4)** in the anterior tuberal nucleus. The third panel contains micrographs showing ARα and ARβ mRNA **(C2,C3)** and AR protein **(C4)** patterns in the periventricular nucleus of the posterior tuberculum. The fourth panel contains micrographs showing ARα and ARβ mRNA **(D2,D3)** and AR protein **(D4)** in the semicircular torus (ST). The bottom panel shows micrographs of ARα and ARβ mRNA **(E2,E3)** and AR protein **(E4)** patterns in the periaqueductal gray. Scale bars = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]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nLT
TPp
Cn
LR
Dn
A2
vTn
A3
vTn
A4
vTn
B2
aTn
B3
aTn
B4
aTn
C2
TPp
C3
TPp
C4
TPp
D2
ST
D3
ST
D4
ST
E2
PAG
E3
PAG
E4
PAG

Figure 3

interest. The degree of shading qualitatively represents the density of mRNA expression in that region. Unless specifically stated, all descriptions of protein-immunoreactivity refer to nuclear staining. In the maps for PR and ERα, mRNA is shown as gray shading on the right half of each representative section. Since teleosts have two subtypes of the AR (ARα and ARβ) and ERβ (ERβa and ERβb), the alpha subtypes are displayed on the left portion of each representative section while the beta subtype is depicted on the right. The antibodies available for either AR or ERβ do not allow us to distinguish these subtypes via IHC. The general patterns shown here are representative of both dominant and subordinate males (notwithstanding possible quantitative differences, which we do not investigate here). Overall, the mRNA detection via in situ hybridization and protein immunohistochemistry staining showed high congruence. Control slides that include omitting antibody for immunohistochemistry and hybridizing with sense probes for in situ hybridization showed no specific signal.

Androgen receptor

Robust expression of both ARβ and ARα mRNA and AR protein is seen throughout the telencephalon, diencephalon, and mesencephalic structures of *A. burtoni* (Fig. 2). In general, ARα and ARβ show similar patterns of expression and consistently overlap with AR protein immunoreactivity.

Telencephalon

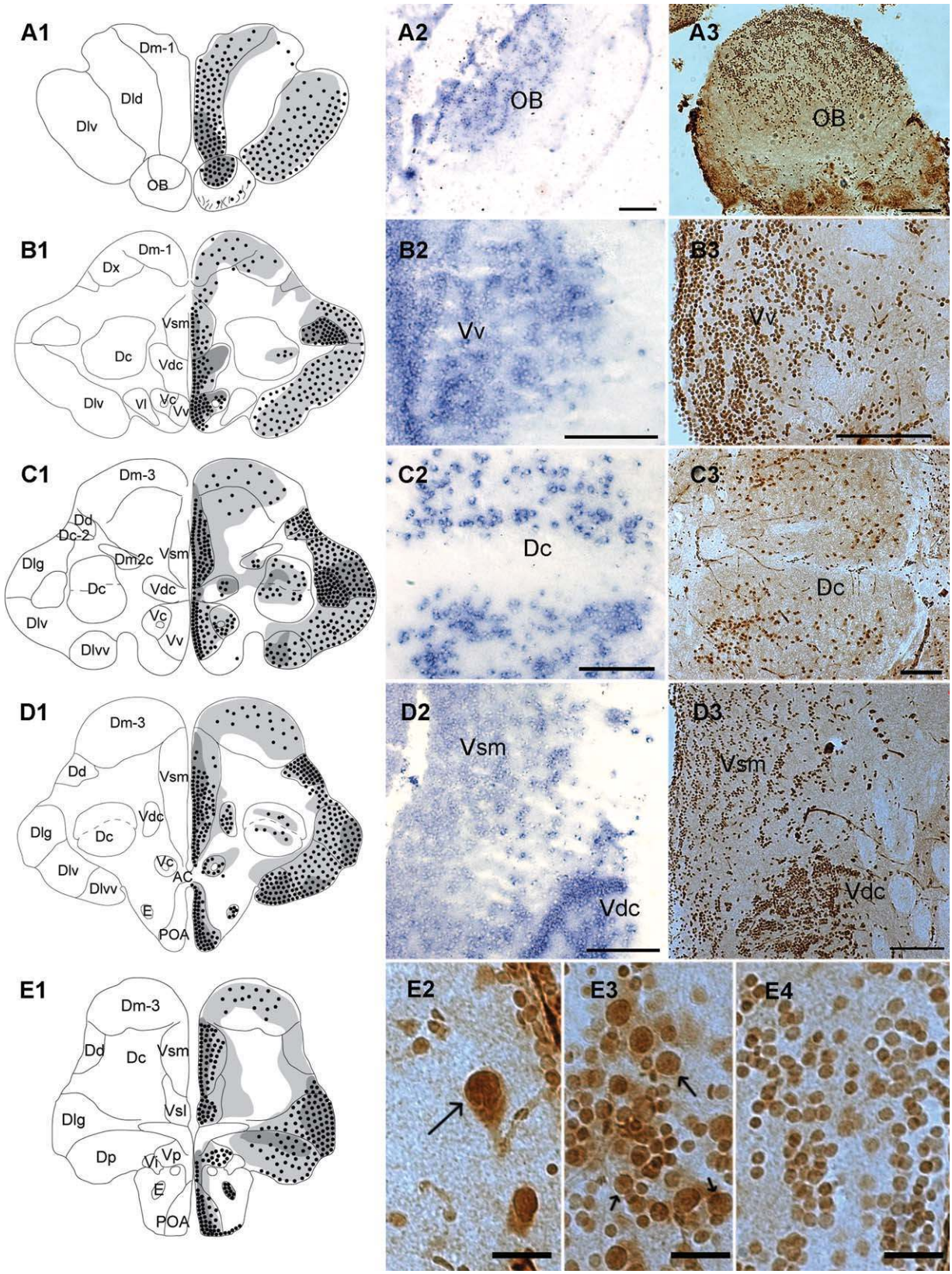
Strong signal for ARα and ARβ mRNA and AR protein is found in discrete parts of the dorsal and ventral telencephalon (Fig. 2). There is robust expression of AR protein and mRNA of both subtypes in the granule cell layer of the olfactory bulb (OB, Fig. 2A). However, AR-immunoreactivity is also seen in the glomeruli, although mRNA is not expressed here. In the dorsal telencephalon there is signal of mRNA of both subtypes and AR-immunoreactive cells including the central, lateral, medial, and posterior parts (Dc, Dl, Dm, and Dp, respectively). Subdivisions within these regions with heavy staining are the granular part of Dl and the ventral part of the ventral Dl (Dlg and Dlvv). AR staining is nearly absent in the dorsal part of Dl (Dld). There are two distinct cell groups in Dc with light staining (Fig. 2C). The patterns of AR subtypes within the dorsal telencephalon vary, with ARβ staining being more restricted than ARα. Region Dx has AR protein but only ARβ staining. In the rostral part of Dd only ARβ mRNA is expressed, but in the more caudal regions of Dd both subtypes are present. Within the ventral telencephalon there is staining of both AR subtypes and AR-immunoreactive cells within the ventral, central, dorsal, and supracommissural parts (Vv, Vc, Vd, and Vs, respectively; Fig. 2B–E). There is good overlap between staining of both subtypes of AR and protein immunoreactivity in the ventral telencephalon with the exception of Vdc, which has more ARα mRNA than ARβ. Finally, AR protein and mRNA of both subtypes are present in the entopeduncular nucleus (E).

The preoptic area (POA) has very heavy staining of AR mRNA subtypes and AR protein. The teleost POA has three cell populations that play distinct roles in modulating behavior (Greenwood et al., 2008): parvocellular, magnocellular, and gigantocellular neurons. AR protein immunoreactivity was observed in each of these cell groups (Fig. 2E2–4).

Diencephalon and mesencephalon

The pattern of both AR subtype mRNA expression and cell-immunoreactivity for AR show extensive overlap, similar to patterns seen in the telencephalon, although the diencephalic AR patterns are more diffuse than those seen in the telencephalon (Fig. 3). Perhaps the most striking pattern seen in the diencephalon and some mesencephalic structures is the intense mRNA staining and protein immunoreactivity of the optic tectum (OT) and longitudinal torus (LT). Caudal to the POA, ARα and ARβ mRNA hybridization and AR protein immunoreactivity is found in the habenula (H) and the ventromedial thalamic nucleus (VM). Several periventricular pretectal nuclei show AR staining including the rostral, dorsal, and ventral regions (PPr, PPd, and vPPn, respectively). AR protein and mRNAs are present within the prethalamic nucleus (PN), which lies ventrolateral to VM. AR mRNA and protein is abundant in the anterior ventral hypothalamic nuclei including the ventral and anterior tuberal regions (vTn and aTn, respectively; Fig. 3A,B) and the periventricular hypothalamic regions including

Figure 4. Distribution of ERα mRNA and protein in the telencephalon. Representative sections of the telencephalon are presented as the first image in each panel with ERα protein shown as dots and mRNA shown as shading on the right side of the brain. The density of dots indicating protein corresponds to the density of cells positive for ERα-immunoreactivity. The degree of shading for mRNA corresponds to the density of expression. The micrographs in the top panel show ERα mRNA **(A2)** and ERα protein **(A3)** in the olfactory bulb. Micrographs in the second panel show labeling of ERα mRNA **(B2)** and protein **(B2)** in Vv. The third panel shows micrographs of ERα mRNA **(C2)** and protein **(C3)** in Dc. The fourth panel shows micrographs of ERα mRNA **(D2)** and protein **(D3)** in Vdc and Vsm. The bottom panel shows ERα protein in gigantocellular **(E2)**, magnocellular **(E3)**, and parvocellular **(E4)** cells in the POA. Scale bars = 100 μm except E2–E4 = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]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B2
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B3
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Dm-3
Dd
Dc
Vsm
Vsl
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Dp
Vl
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Figure 4

the ventral hypothalamus (VH), lateral hypothalamic nucleus (LHn), and the dorsal hypothalamus (DH). Lateral to these regions, staining is also found within the inferior lobe including the central (Cn) and diffuse nuclei (Dn). Abundant AR expression is found throughout the extent of the periventricular nucleus of the posterior tuberculum (TPp; Fig. 3C) and the thalamic region, central posterior thalamic nucleus (CP). A weak signal of both AR mRNA subtypes and light protein immunoreactivity is found within the glomerular nucleus (Gn). AR staining is also present within the lateral torus (nLT). Both mRNA and protein are expressed within the semicircular torus (ST, Fig. 3D) and the periaqueductal gray (PAG; Fig. 3E). In the caudal diencephalon the preglomerular commissural nucleus (PGCn) and the mammillary body (MB) also contain both AR mRNAs and protein (not shown). Finally, strong signal of AR protein and mRNA of both subtypes is found within the pituitary (Fig. 3C1).

Estrogen receptor alpha

ERα mRNA staining and protein immunoreactivity are found throughout the telencephalon, diencephalon, and some mesencephalic structures overall showing a high degree of overlap.

Telencephalon

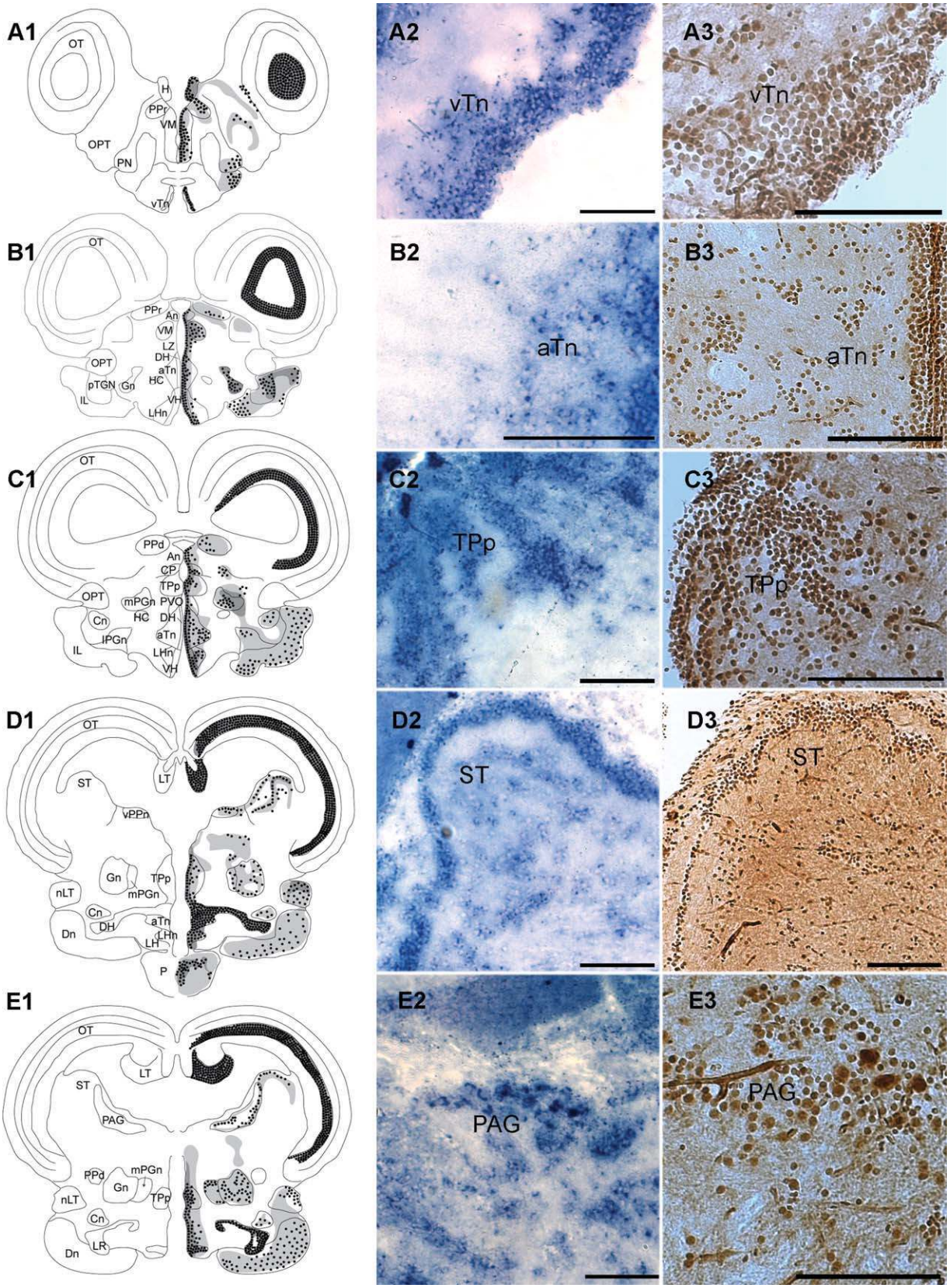
An abundance of ERα is present within the dorsal and ventral telencephalon (Fig. 4). There is a pronounced expression of both mRNA and protein within the granule layer of the olfactory bulb (OB, Fig. 4A). ERα protein is also found within the glomeruli, although mRNA expression is not seen here. Within the dorsal telencephalon, ERα is present in Dc, Dd, Dl, and Dm, with the strongest ERα mRNA signal and densest cell-immunoreactivity found in Dd and Dlvv. There is very little staining in Dld. There are two distinct cell groups within Dc that have light staining of both mRNA and protein (Fig. 4C). Within the ventral telencephalon, ERα mRNA and protein is present within the posterior part (Vp) as well as Vc, Vd, Vp, Vs, and Vv (Fig. 5B–E). Interestingly, ERα mRNA and protein are absent within Vl, making it the only steroid hormone receptor absent within this part of V. Regions with the heaviest staining for mRNA and highest density of immunoreactive cells are in Vdc, the medial part of Vs (Vsm), and Vv.

Within the POA there was heavy staining of ERα mRNA and a high population of ERα-immunoreactive cells. ERα was present within all three POA cell subtypes: parvocellular, magnocellular, and gigantocellular (Fig. 4E2–4). Finally, ERα mRNA and protein are also present within the entopeduncular nucleus (E).

Diencephalon and mesencephalon

Compared to ERα patterns found in the telencephalon, mRNA and protein patterns are more diffuse in the diencephalon and mesencephalic structures, and similar to the distributions found for the AR subtypes. ERα mRNA and protein continue to show high concordance in these more caudal regions (Fig. 5). Caudal to the POA the VM has abundant ERα mRNA and protein presence. Plentiful ERα staining of both mRNA and protein was also seen in the habenula (H). Ventrolateral to VM, ERα protein and mRNA is abundant within the prethalamic nucleus (PN). Within the periventricular pre-tectal nuclei, ERα is found within the PPr, PPd, and vPPn. In the anterior ventral hypothalamic nuclei, ERα is found within both the vTn and aTn (Fig. 5A,B) as well as in the DH, VH, and LHn. Interestingly, there is intense expression of ERα mRNA as well as ERα protein immunoreactivity in the OT and the LT, similar to that seen with AR. ERα is highly expressed throughout the TPp (Fig. 5C) and in the thalamic nuclei, CP. Staining is also found lateral to these regions in the inferior lobe including the Cn and Dn. Dorsal to Cn and Dn, the nLT also contains ERα mRNA and protein. Light mRNA and protein staining is found within the Gn. Dorsal and lateral to the thalamus, ERα mRNA and protein is present in ST (Fig. 5D). The PAG also contains ERα mRNA and protein (Fig. 5E). Caudally in the diencephalon, the mammillary body (MB) and preglomerular commissural nucleus (PGCn) both express ERα mRNA and protein (not shown). Finally, the pituitary shows intense expression

Figure 5. Distribution of ERα mRNA and protein in the diencephalon and mesencephalon. Representative sections of the diencephalon and some mesencephalic structures are depicted in the left column with ERα protein represented by dots and mRNA as gray shading. The density of dots indicating protein corresponds to the density of cells positive for ERα-immunoreactivity. The degree of shading for mRNA corresponds to the density of expression. Micrographs in the top row show ERα mRNA **(A2)** and protein **(A3)** in the ventral tuberal nucleus. Micrographs in the second row show ERα mRNA **(B2)** and protein **(B3)** in the anterior tuberal nucleus. The third panel contains micrographs showing ERα mRNA **(C2)** and protein **(C3)** patterns in the periventricular nucleus of the posterior tuberculum. The fourth panel contains micrographs showing ERα mRNA **(D2)** and protein **(D3)** in the semicircular torus (ST). The bottom panel shows micrographs of ERα mRNA **(E2)** and protein **(E3)** patterns in the periaqueductal gray (PAG). Scale bars = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]


A1
OT
H
PPr
VM
OPT
PN
vTn
B1
OT
PPr
An
VM
LZ
DH
aTn
HC
VH
LHn
OPT
pTGN
Gn
IL
C1
OT
PPd
An
CP
TPp
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B2
aTn
B3
aTn
C2
TPp
C3
TPp
D2
ST
D3
ST
E2
PAG
E3
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Figure 5

of ERα mRNA and heavy staining of protein immunoreactivity.

Estrogen receptor beta

ERβ is abundant throughout the telencephalon, diencephalon, and mesencephalon. Staining of the ERβ mRNA subtypes, ERβa and ERβb, largely overlap, but the ERβb subtype appears more widespread in the telencephalon compared to ERβa. While the other sex steroid hormone receptors in the study are very similar in distribution, ERβ patterns appear the most different from the other sex steroid receptors. Additionally, the ERβ antibody showed either cytosolic or nuclear staining in discrete brain regions. This is similar to ER staining patterns in mammals, where nuclear and/or cytosolic staining is seen throughout development in the pituitary and in adult epithelial cells (Pasqualini et al., 1999; Flynn et al., 2008) These different staining patterns are represented in the distribution maps (Figs. 6, 7) by open circles (cytosolic) or filled circles (nuclear).

Telencephalon

ERβa and ERβb mRNA and protein are found in discrete nuclei of the dorsal and ventral telencephalon (Fig. 6). Interestingly, ERβ cell immunoreactivity differed in nuclear or cytosolic staining in discrete brain regions. Overall, there was very consistent overlap of protein immunoreactivity and mRNA detection of both subtypes. Strong mRNA hybridization of both mRNA subtypes and high levels of protein immunoreactivity are seen in the granule cell layer of the olfactory bulb (OB, Fig. 6A). ERβ protein is also found within fibers of the glomeruli, although mRNA expression is not found here. In the dorsal telencephalon, ERβ protein and both ERβa and ERβb mRNA subtypes are present within Dc, Dl, and Dm. Within these dorsal regions, Dlg has the highest density of immunoreactive cells. ERβ is the only steroid receptor expressed abundantly in Dld; PR is present, yet very sparse (see below). This staining is most likely ERβb, as there is ERβb mRNA staining in this region but not ERβa mRNA (Fig. 6A1). Two distinct cell groups positive for ERβ form within Dc (Fig. 6C). The staining in both Dld and Dc is cytosolic, suggesting that ERβ is not affecting gene transcription within these regions. Within Dl, Dlg has an abundance of ERβ compared to Dlv. Within the ventral telencephalon, ERβ protein and mRNA subtypes are present within Vc, Vd, Vs, and Vv (Fig. 6B–E). While the pattern of cellular localization in the dorsal telencephalon differs between brain regions, all immunoreactive cells within the ventral telencephalon had predominantly nuclear staining. Finally, ERβ protein and mRNA of both subtypes are present in the entopeduncular nucleus (E).

ERβ protein and mRNA was also present within the POA, although staining patterns were more restricted within the POA regions compared to the distributions of AR, ERα, and PR in the POA. In some individuals, ERβ was more abundant within the dorsal portion of the parvocellular POA than the ventral region. ERβ immunoreactivity was also observed in magnocellular and gigantocellular cells (Fig. 6E). Interestingly, within the gigantocellular cells the ERβ staining pattern is different from that of the other nuclear hormone receptors in this study, as there appear to be aggregates of protein surrounding the nucleus (Fig. 6E2). These receptors may be inactivated in that they remain in cytosolic aggregates rather than translocate to the nucleus to modulate gene transcription.

Diencephalon and mesencephalon

ERβ staining in the diencephalon and mesencephalon is more diffuse than in the telencephalon, but more widespread than the other steroid hormone receptors in this study (Fig. 7). There continues to be high overlap between staining patterns of the ERβ mRNA subtypes and ERβ protein immunoreactivity. Caudal to the POA, ERβ protein and mRNA of both subtypes are present within the VM. The prethalamic nucleus (PN), which lies ventrolateral to VM, also contains ERβ protein and mRNA of both subtypes. The habenula (H) also shows heavy straining of ERβ mRNA and protein. ERβa and ERβb mRNA and protein are also found within the OT and the LT. In the anterior ventral hypothalamus, ERβ is present within the vTn and aTn (Fig. 7A,B). ERβ is also

Figure 6. Distribution of ERβ mRNA and protein in the telencephalon. Representative sections of the telencephalon are presented as the first image in each panel with ERβ protein shown as dots on the right side of the brain and ERβa mRNA shown as gray shading on the left and ERβb mRNA as gray shading on the right. Open circles denote cytosolic staining while closed circles indicate nuclear localization. The density of dots indicating protein corresponds to the density of cells positive for ERβ-immunoreactivity. The degree of shading for mRNA corresponds to the density of expression. The micrographs in the top panel show ERβa and ERβb mRNA **(A2,A2)** and ERβ protein **(A4)** in the olfactory bulb. Micrographs in the second panel show labeling of ERβa and ERβb mRNA **(B2,B3)** and ERβ protein **(B4)** in Vv. The third panel shows micrographs of ERβa and ERβb mRNA **(C2,C3)** and ERβ protein **(C4)** in Dc. The fourth panel shows micrographs of ERβa and ERβb mRNA **(D2,D3)** and ERβ protein **(D4)** in Vdc and Vsm. The bottom panel shows ERβ protein in gigantocellular **(E2)**, magnocellular **(E3)**, and parvocellular **(E4)** cells in the POA. Scale bars = 100 μm except E2–E4 = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

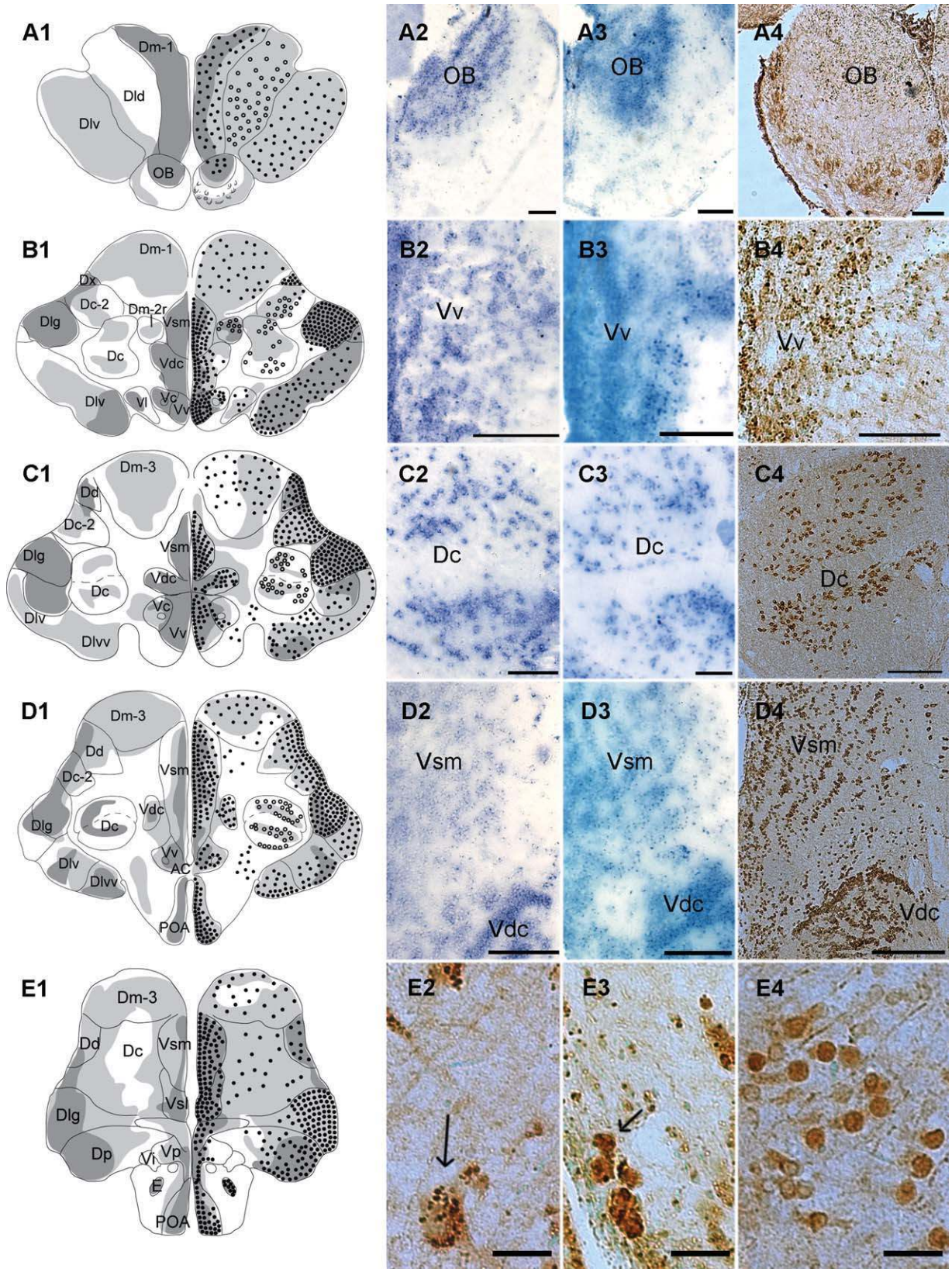
A1
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Figure 6

present within the DH, LHn, and VH, and in the periventricular pretectal nuclei PPr, PPd, and vPPn. The thalamic nucleus, CP, also shows ERβ staining. The TPp shows staining of both ERβ mRNA subtypes and protein (Fig. 7C). The inferior lobe also contains ERβ in the Cn and Dn nuclei. Staining of ERβa and ERβb mRNAs and protein-immunoreactive cells was observed within Gn and nLT. ERβ mRNA and protein is present in the ST (Fig. 7D) and ventromedial to the ST, the PAG also contains ERβ mRNA and protein (Fig. 7E). ERβ mRNAs and protein are also found in the caudal diencephalic nuclei, mammillary body (MB, not shown) and preglomerular commissural nucleus (PGCn, not shown). Finally, in the pituitary ERβa and ERβb mRNAs are present throughout, whereas ERβ protein immunoreactivity is more heterogeneous.

Progesterone receptor

To our knowledge, we present here the first detailed description of PR in the telencephalon, diencephalon, and mesencephalon of any teleost, although preoptic and hypothalamic regions have recently been described in zebrafish (Hanna et al., 2010). PR is abundant in the telencephalon and diencephalon, and mRNA and protein show high congruence.

Telencephalon

An abundance of PR is found within distinct regions of the dorsal and ventral telencephalon (Fig. 8). There is robust staining of PR mRNA and protein within the granule cell layer of the olfactory bulb (OB, Fig. 8A). Like the other steroid hormone receptors, PR protein is found within the glomeruli, but mRNA is not expressed here. Within the dorsal telencephalon, PR protein and mRNA was present in Dc, Dd, Dl, and Dm, and more sparsely in Dld. The heaviest staining for mRNA and highest density of immunoreactive cells is found within Dlg. PR mRNA and protein cluster into two distinct groups within Dc (Fig. 8C). Within the ventral telencephalon, PR protein and mRNA are present within Vc, Vd, Vs, and Vv (Fig. 8B–E). Regions with the highest density of PR immunoreactive cells are the Vv and Vsm.

There is a very strong PR mRNA signal and a very high density of PR-immunoreactive cells within the POA. PR is found within parvocellular, magnocellular, and gigantocellular cells (Fig. 8E). Finally, the entopeduncular nucleus (E) contains both PR mRNA expression and protein immunoreactivity.

Diencephalon and mesencephalon

PR mRNA and protein is generally more diffuse in the diencephalon and mesencephalon compared to the telencephalon (Fig. 9). As with patterns seen in the telencephalon, mRNA and protein patterns show high concordance. One of the most striking staining patterns is the robust mRNA and protein signal in the OT and LT, similar to the other sex steroid receptors. Caudal to the POA, PR mRNA and protein is found within the VM. Ventrolateral to VM, the prethalamic nucleus (PN) contains both PR protein and mRNA. The habenula also contains PR mRNA and protein. Within the periventricular pretectal nuclei, PR is present within PPr, PPd, and vPPn. PR mRNA and protein signal is abundant within the anterior ventral hypothalamic nuclei, including the vTn and aTn (Fig. 9A,B) and the DH, VH and LHn. PR mRNA and protein is consistently found throughout the TPp (Fig. 9C) and in the thalamic CP. PR staining is also present within the semicircular torus ST (Fig. 9D) and PAG (Fig. 9E). The lateral torus (nLT) contains both mRNA and protein. In the ventral lateral portion of the hypothalamus, staining is found in the inferior lobe including the Cn and Dn. Light staining of PR protein and mRNA is also found in Gn. More caudally in the diencephalon, mammillary body (MB) and preglomerular commissural nucleus (PGCn) also contain PR mRNA and protein (not shown). Finally, PR mRNA and protein are abundant within the pituitary.

DISCUSSION

We have shown here for males of the African cichlid fish, *A. burtoni*, that steroid hormone receptors are widely

Figure 7. Distribution of ERβ mRNA and protein in the diencephalon and mesencephalon. Representative sections of the diencephalon and some mesencephalic structures are depicted in the left column with ERβ protein represented by dots on the right, ERβa mRNA as gray shading on the left, and ERβb mRNA as gray shading on the right. Open circles denote cytosolic staining while closed circles indicate nuclear localization. The density of dots indicating protein corresponds to the density of cells positive for ERβ-immunoreactivity. The degree of shading for mRNA corresponds to the density of expression. Micrographs in the top row show ERβa and ERβb mRNA **(A2,A3)** and ERβ protein **(A4)** in the ventral tuberal nucleus. Micrographs in the second row show ERβa and ERβb mRNA **(B2,B3)** and AR protein **(B4)** in the anterior tuberal nucleus. The ovoid structure in B2 is a fiber tract that appears alongside the aTn. The third panel contains micrographs showing ERβa and ERβb mRNA **(C2,C3)** and ERβ protein **(C4)** patterns in the periventricular nucleus of the posterior tuberculum. The fourth panel contains micrographs showing ERβa and ERβb mRNA **(D2,D3)** and ERβ protein **(D4)** in the semicircular torus (ST). The bottom panel shows micrographs of ERβa and ERβb mRNA **(E2,E3)** and ERβ protein **(E4)** patterns in the periaqueductal gray (PAG). Scale bars = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

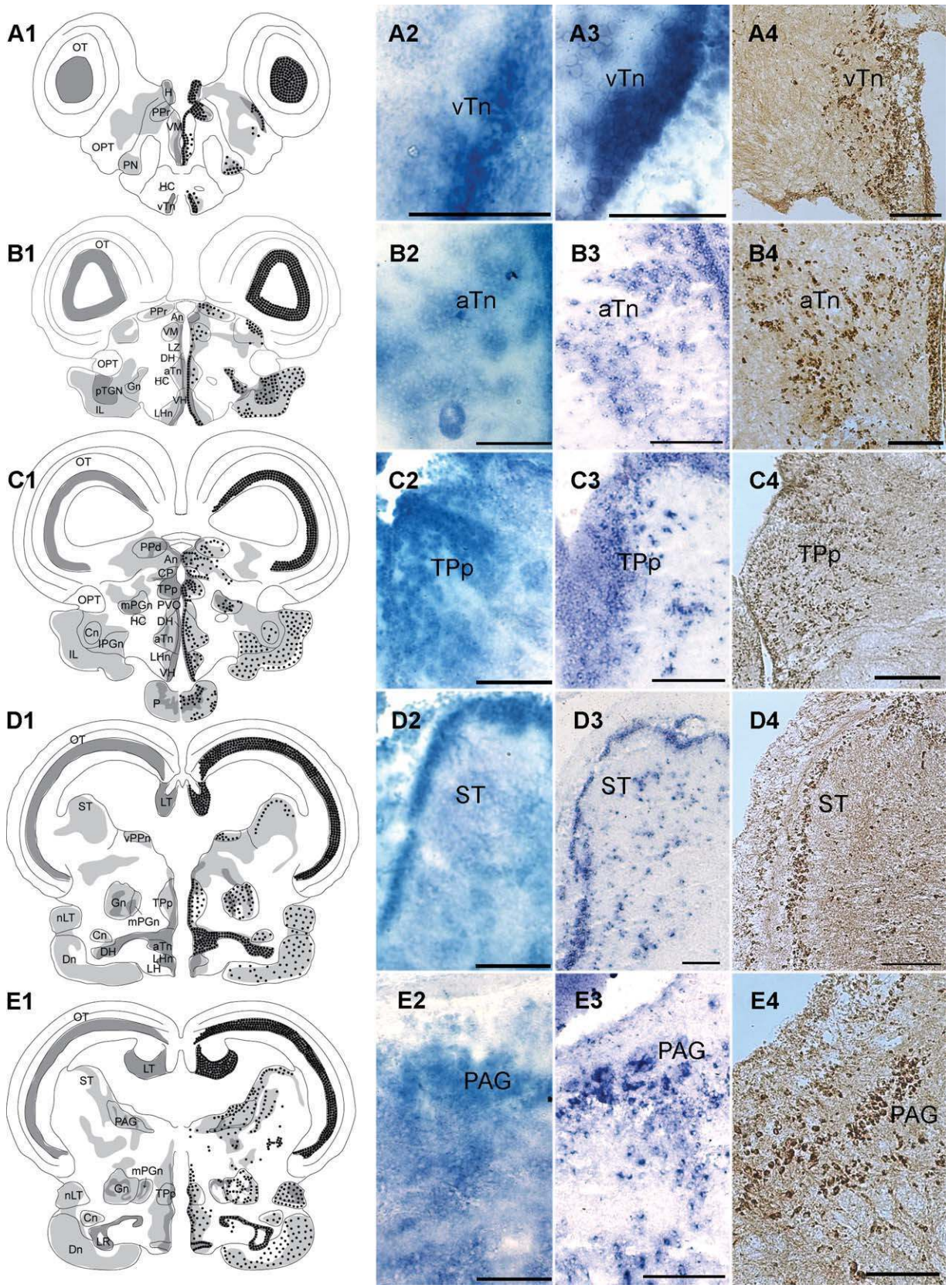
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Figure 7

distributed throughout the telencephalon and diencephalon, and have elucidated which regions of the brain are likely targets of sex steroid modulation. We find excellent correspondence between mRNA expression patterns and protein immunoreactivity of each sex steroid hormone receptor. Further, we find sex steroid hormone receptors in neural circuits that are known across vertebrates to modulate social behaviors and/or process the salience/rewarding properties of a stimulus. There is remarkable overlap in the presence of all steroid hormone receptors within many brain nuclei. Variation occurs in the pallial area Dld, where only ERβ and PR are expressed (the latter quite sparsely), and in subpallial area Vl, which lacks ERα expression. Areas Vp and Dd lack expression of ARα and ARβ, respectively. Interestingly, the subpallial area Vi, which contains sex steroid receptors in midshipman fish (Forlano et al., 2010), is the only fore- and midbrain region examined where we did not find a single sex steroid hormone receptor. Finally, while all sex steroid hormones were abundant in the granule layer of the OB, only protein was found in the glomerular layer. This discrepancy could be due to sensitivity in mRNA staining or that the protein is translocated to other regions away from the source of translation.

In the following we compare the findings presented here for the cichlid fish *A. burtoni* with previously published results in other species of teleosts as well as tetrapods. Variation within and across species could possibly result from differences in sex and reproductive state in addition to evolutionary divergence and issues related to homology of brain structures. It is important to note, however, that—as a consequence of organizational and/or activational control—variation in sex steroid receptor expression (between sexes; among fixed or plastic alternative phenotypes; or across seasons) typically manifests itself as quantitative differences in expression level, and not qualitatively as presence or absence (Godwin and Crews, 1995; Burmeister et al., 2007). Finally, discrepancies could also result from differences in the techniques used, as most previous studies only detected mRNA and the signal of the expression can vary based on development times.

Neuroanatomical distribution of sex steroid hormones compared with other teleosts

Androgen receptor

AR is widely distributed throughout the *A. burtoni* telencephalon and diencephalon, and mRNA and protein patterns show very high congruence. ARα and ARβ mRNA expression patterns have previously been examined in this species (Harbott et al., 2007), although only in telencephalon and hypothalamic areas. Additionally, the present study for the first time determined AR protein distribution in a percomorph fish. The brain regions positive for ARα and ARβ expression in this present study are consistent with those reported in Harbott et al. (2007).

The wide distribution pattern of AR protein and the mRNA subtypes shows high overlap with mRNA distributions described for the midshipman (Forlano et al., 2010) and zebrafish (Gorelick et al., 2008) and protein distributions described in goldfish (Gelinas and Callard, 1997). The studies in zebrafish and midshipman did not distinguish between ARα and ARβ mRNA subtypes. As in *A. burtoni*, AR protein is expressed within the olfactory bulb of goldfish, but mRNA was not detected within the olfactory bulb of midshipman or zebrafish. Within the dorsal telencephalon (D), the central (Dc) and lateral (Dl) subdivisions contain AR protein in goldfish, only Dl contains AR mRNA in zebrafish, and Dc and medial subdivision of D (Dm) contain AR mRNA in the midshipman, while *A. burtoni* show mRNA of both subtypes and AR protein in these regions. Comparing *A. burtoni* with these three species, only *A. burtoni* shows AR presence within the posterior division of D (Dp). Within the ventral telencephalon (V), AR protein is found within the goldfish dorsal (Vd) and lateral (Vl) subdivisions of V, and AR mRNA is present within the supracommissural (Vs), postcommissural (Vp), dorsal (Vd), and intermediate (Vi) subdivisions of V in the midshipman. Unexpectedly, no subpallial areas were reported to contain AR in zebrafish. Both AR protein and mRNA subtypes are expressed within all of the ventral telencephalon regions previously reported in the other teleost species with the exception of Vi. Surprisingly, AR is not found within the ventral subdivision in the ventral telencephalon (area Vv) of the above teleost species,

Figure 8. Distribution of PR mRNA and protein in the telencephalon. Representative sections of the telencephalon are presented as the first image in each panel with PR protein shown as dots and mRNA shown as shading. The density of dots indicating protein corresponds to the density of cells positive for PR-immunoreactivity. The degree of shading for mRNA corresponds to the density of expression. The micrographs in the top panel show PR mRNA **(A2)** and PR protein **(A3)** in the olfactory bulb. Micrographs in the second panel show labeling of PR mRNA **(B2)** and protein **(B2)** in Vv. The third panel shows micrographs of PR mRNA **(C2)** and protein **(C3)** in Dc. The fourth panel shows micrographs of PR mRNA **(D2)** and protein **(D3)** in Vdc and Vsm. The bottom panel shows PR protein in gigantocellular **(E2)**, magnocellular **(E3)**, and parvocellular **(E4)** cells in the POA. Scale bars = 100 μm except E2–E4 = 20 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

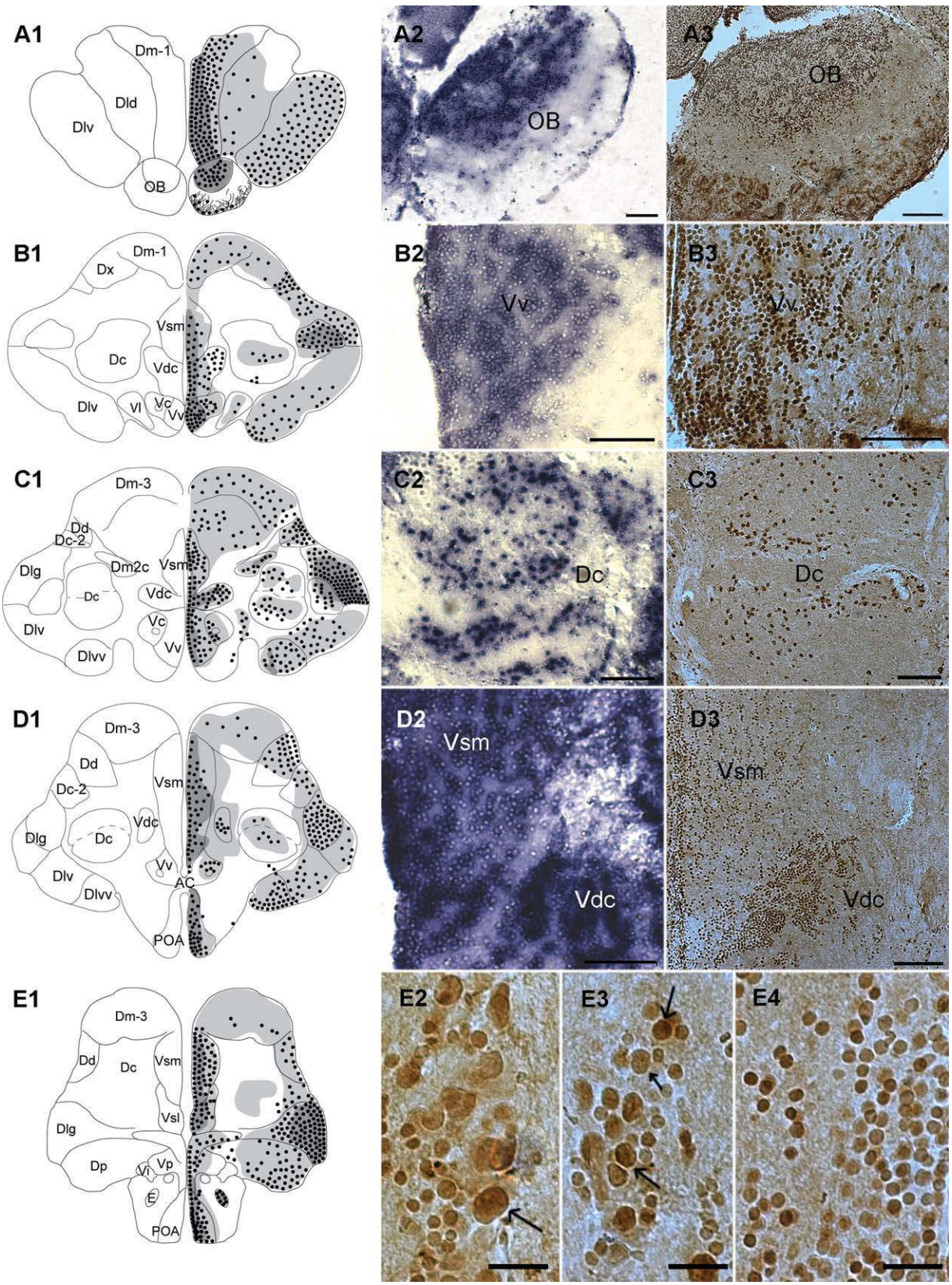
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Figure 8

although we find robust expression and protein presence in this region in the present study, consistent with previous studies of AR in *A. burtoni* (Harbott et al., 2007). AR mRNA of both subtypes and protein is found in the preoptic area of *A. burtoni*, zebrafish, midshipman, and goldfish. We also found an abundance of AR in the optic tectum, tuberal nuclei, and hypothalamic regions, consistent with the previous reports in goldfish, zebrafish, and the midshipman.

Estrogen receptor alpha

ERα is also widely distributed throughout the *A. burtoni* telencephalon and diencephalon, much more so than previously published distributions in other teleost species, although most of these studies describe mRNA only and not protein. ERα mRNA distributions have been described in the midshipman (Forlano et al., 2005), Atlantic croaker (Hawkins et al., 2005), rainbow trout (Menuet et el., 2001), zebrafish (Menuet et al., 2002), and eelpout (Andreassen et al., 2003). In general, ERα is consistently found within the ventral regions of the ventral telencephalon (Vv), the preoptic area, and the hypothalamus; however, comparing across teleosts is difficult as many of these studies report only neuroendocrine regions of the brain, with the exception of the midshipman (Forlano et al., 2005). Unlike the midshipman, ERα is found within the lateral portion of the dorsal telencephalon (D1) and the dorsal and caudal regions of the ventral telencephalon (Vd and Vc) in *A. burtoni*. *A. burtoni* and the midshipman have very similar distribution patterns of ERα in ventral hypothalamus, thalamus, and brainstem regions including the periaqueductal gray.

Estrogen receptor beta

ERβ is widely distributed throughout the *A. burtoni* telencephalon and diencephalon, where the ERβb mRNA subtype appears to be more widespread than ERβa. Distribution of ERβa and ERβb mRNA subtypes have been described in the Atlantic croaker (Hawkins et al., 2005), sea bass (Muriach et al., 2008), and zebrafish (Menuet et al., 2002), although to our knowledge no studies have examined protein distribution. Studies in sea bass and zebrafish have described ERβ mRNA expression patterns in the POA, hypothalamus, and posterior tuberculum, in which both ERβ subtypes are expressed, similar to *A. burtoni*. In contrast to the Atlantic croaker, where ERβb, and not ERβa, is expressed in the posterior tuberculum, both *A. burtoni* and zebrafish express both subtypes within this region. Our results contrast with those from sea bass, where ERβa was expressed in the ventral telencephalon and the POA, but ERβb expression was restricted to the POA.

Progesterone receptor

We have cloned a single PR mRNA transcript from *A. burtoni* and found no evidence for additional PR genes. The Japanese eel likely has two PR subtypes from two different genes (Ikeuchi et al., 2002). However, other ray-finned fishes such as zebrafish, stickleback, and medaka (which all have sequenced genomes available) appear to only have one (Hanna et al., 2010), suggesting either a possible gene duplication in the lineage leading to eels (Anguilliformes) or the deletion of the gene at the base of euteleosts. It is thus unlikely that *A. burtoni* has more than one PR gene.

As with the other sex steroid receptors, we found that PR mRNA and protein have exceptional concordance and are widely distributed throughout the telencephalon and diencephalon. The distribution of PR mRNA and protein in the hypothalamus and preoptic area has been recently described in zebrafish (Hanna et al., 2010). PR is expressed in the same regions of the telencephalon in both zebrafish and *A. burtoni*. The only region of the ventral telencephalon (V) described by Hanna et al. (2010) was the postcommissural subdivision of the V (Vp), which expresses PR, similar to *A. burtoni*. Regions of the zebrafish brain in the dorsal telencephalon (D) were the medial, lateral, and posterior divisions of D (Dm, Dl, and Dp), in which PR is abundant in both species. In the diencephalon of both zebrafish and *A. burtoni*, PR was also expressed in the periventricular nucleus of the posterior tuberculum (TPp) and in the thalamic central posterior nucleus (CP). Finally PR is expressed in the zebrafish dorsal, ventral, and lateral periventricular hypothalamic areas (DH, VH, and LHn, respectively) and in the anterior tuberal nucleus (aTn), similar to *A. burtoni*.

Figure 9. Distribution of progesterone receptor mRNA and protein in the diencephalon and mesencephalon. Representative sections of the diencephalon and some mesencephalic structures are depicted in the left column with PR protein represented by dots and mRNA as gray shading. The density of dots indicating protein corresponds to the density of cells positive for PR-immunoreactivity. The degree of shading for mRNA corresponds to the density of expression. Micrographs in the top row show PR mRNA **(A2)** and protein **(A3)** in the ventral tuberal nucleus. Micrographs in the second row show PR mRNA **(B2)** and protein **(B3)** in the anterior tuberal nucleus. The third panel contains micrographs showing PR mRNA **(C2)** and protein **(C3)** patterns in the periventricular nucleus of the posterior tuberculum. The fourth panel contains micrographs showing PR mRNA **(D2)** and protein **(D3)** in the semicircular torus (ST). The bottom panel shows micrographs of PR mRNA **(E2)** and protein **(E3)** patterns in the periaqueductal gray (PAG). Scale bars = 100 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

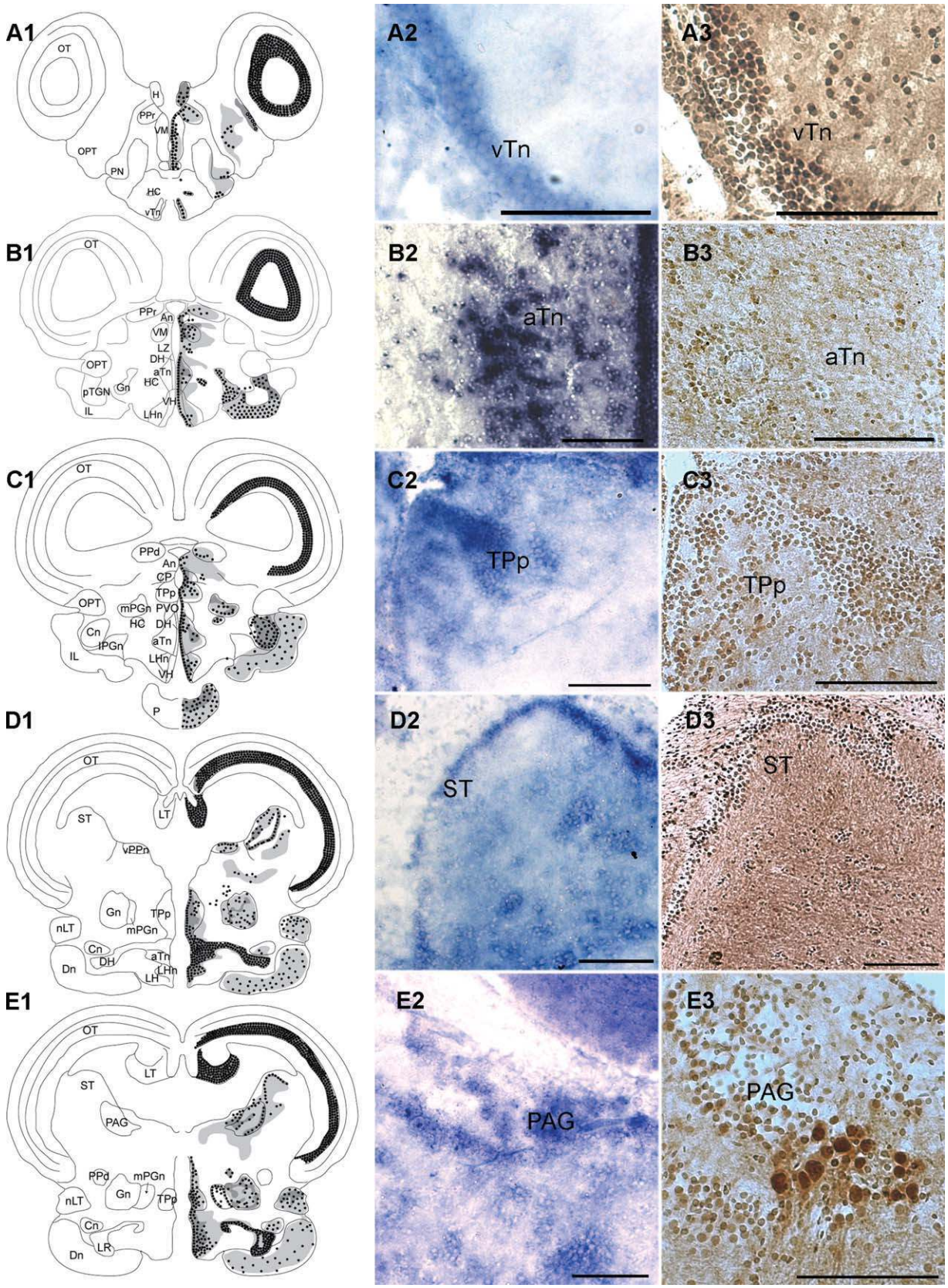
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Figure 9

Functional considerations of sex steroid hormone receptors in the teleost brain

Various lesion and stimulation studies have pointed to the POA as a brain region that regulates aggressive and reproductive behaviors in teleosts, consistent with findings in other vertebrates (Wheeler and Crews, 1978; de Jonge et al., 1989). Electrical stimulation of the POA increases courtship behavior and decreases aggressive displays in *Lepomis* sunfish males (Demski and Knigge, 1971). It is obviously advantageous to a male to decrease aggressive displays while courting a female (Miller, 1963). Ablation of the POA decreases the spawning reflex in male killifish, *Fundulus heteroclitus* (Macey et al., 1974). We have found all sex steroid hormone receptors within all three cell groups (parvocellular, magnocellular, and gigantocellular) in the POA (Figs. 2E, 4E, 6E, 8E). Previous work by Greenwood et al. (2008) in *A. burtoni* has suggested that these three cell groups may differentially regulate social dominance, as dominant males have higher AVT expression in the gigantocellular subregion compared to subordinate males. However, subordinate males have higher AVT expression in the parvocellular portion, while AVT expression in the magnocellular portion does not differ with social status. Future studies utilizing quantitative in situ hybridization for sex steroid receptors in the different subpopulations of the POA will provide us insight into whether these receptors are differently modulating social dominance in a region-specific manner.

Also functionally important as a social signal to some male teleost fish, including *A. burtoni*, is the production of body coloration. Compared with subordinate males, dominant *A. burtoni* males show bright nuptial coloration and also have higher levels of circulating testosterone (Parikh et al., 2006a; Greenwood et al., 2008). Functional studies of the neural control of coloration have shown that electrical stimulation of the ventral ST evokes agonistic color responses in *Lepomis* sunfish males (Demski, 1969). We have shown here that all sex steroid hormone receptors are present within the ST, including the androgen receptors, which supports the hypothesis that gonadal hormones also play a role in generating and regulating teleost fish color patterns.

Comparison of sex steroid hormone distributions to tetrapods

The structure of the teleost brain has long been a source of confusion for neuroanatomists, but as evidence accumulated that the brain develops via eversion of the neural tube, rather than inversion as in tetrapods and other nonteleost fishes, much progress has been made in the mapping of the teleost brain and assigning these brain regions putative homologies (Yamamoto et al., 2007). Homologies between brain regions in teleosts and other vertebrates are emerging not only through developmental studies, but also through lesion, neurochemistry, hodology studies (Rink and Wullimann, 2001, 2002; Portavella et al., 2002; Wullimann and Mueller, 2004; Northcutt, 2006, 2008; Bruce and Braford, 2009). Understanding these (partial) homology relationships across vertebrates will be necessary for reconstructing the evolution of two neural circuits that are considered crucial in the regulation of social behavior and the evaluation of rewarding stimuli, respectively: Newman's social behavior network (Newman, 1999) and the mesolimbic dopaminergic system (Wise, 2002). The homologies suggested here for relating various fore- and midbrain regions in teleosts and tetrapods are based on a multitude of considerations, and should thus still be considered tentative.

The social behavior network that was originally described in mammals has now been expanded to other vertebrate classes (Crews, 2003; Goodson, 2005). The brain regions in the network contain mostly hypothalamic regions, mediate social behavior, and express steroid hormone receptors. The nodes of this network include the preoptic area (POA), anterior hypothalamus, ventromedial hypothalamus, medial amygdala (MeAMY) and bed nucleus of the stria terminalis (BNST), periaqueductal gray, and the lateral septum (LS). These regions contain steroid hormone receptors in every vertebrate class studied including reptiles (Young et al., 1994; Moga et al., 2000; Tang et al., 2001; Rosen et al., 2002), amphibians (Meglio et al., 1987; Guerriero et al., 2005), birds (Sterling et al., 1987; Balthazart et al., 1998; Foidart, 1999; Belle and Lea, 2001), and mammals (Kato et al., 1994; Osterlund et al., 1998; Murphy et al., 1999; Roselli et al., 2001; Zhang et al., 2002; Holmes et al., 2008). The only exceptions seem to be in the periaqueductal gray, where ER is absent in reptiles and PR is absent in birds. The putative homologs of these brain regions in teleosts are the supracommissural region of the ventral telencephalon (Vs) as the homolog of the MeAMY/BNST (Northcutt, 1995), the ventral and lateral nuclei of the ventral telencephalon (Vv and Vl) as the homolog of the lateral septum (Wullimann and Müller, 2004), the POA as the homolog to the mammalian medial preoptic area (Moore and Lowry, 1998), the ventral tuberal region as the homolog to the anterior hypothalamus (Kittelberger et al., 2006), the anterior tuberal region as the homolog of the ventromedial hypothalamus (Forlano et al., 2005), and the PAG as the mammalian periaqueductal gray (Forlano et al., 2001). We have shown here that steroid hormone receptors are expressed in each of these brain regions in *A. burtoni*, providing neurochemical evidence in support of these suggested homologies in the social behavior network,

although further manipulative and behavioral studies are still necessary.

The midbrain dopaminergic system consists of the ventral tegmental area, which projects to many forebrain nuclei in what has been described as the "reward" system and is important for reinforcing learned behavior (Young and Wang, 2004). Regions that receive input from this dopaminergic system include the basolateral amygdala, hippocampus, nucleus accumbens, ventral pallidum, striatum, BNST, and the lateral septum. Most of these brain nuclei contain steroid hormone receptors in reptiles (Young et al., 1994; Moga et al., 2000; Tang et al., 2001), amphibians (di Meglio et al., 1987; Guerriero et al., 2005), birds (Sterling et al., 1987; Balthazart et al., 1998; Foidart et al., 1999; Belle and Lea, 2001), and mammals (Kato et al., 1994; Osterlund et al., 1998; Murphy et al., 1999; Zhang et al., 2002; Holmes et al., 2008). However, there are some exceptions, as PR is not present (or has not been reported) within the avian amygdaloid complex or in the VTA; ER is not present (or has not been reported) within the nucleus accumbens, striatum, VTA, or amygdaloid complex of reptiles, and AR is not present (or has not been reported) in the VTA of reptiles or within the striatum of mammals. The putative teleost homologies to these forebrain nuclei are more contentious than those of the social behavior network. The putative homologies are as follows: the medial division of the dorsal telencephalon (Dm) as a putative homolog to the mammalian lateral amygdala (Portavella et al., 2002; Northcutt, 2006), the lateral region of the dorsal telencephalon (Dl) as a putative homolog to the mammalian hippocampus (Portavella, 2004; Northcutt, 2006), the ventral region of the ventral telencephalon (Vv) is a putative homolog to the mammalian nucleus accumbens (Northcutt, 1995; Braford, 2009), the dorsal and central regions of the ventral telencephalon (Vc/Vd) is a putative homolog to the vertebrate striatum (Wullimann and Rink, 2002), the postcommissural region of the ventral telencephalon (Vp) is a putative homolog to the basal amygdala (Nieuwenhuys and Meek, 1990), and the posterior tuberculum (TPp) has been suggested to be at least functionally equivalent (Rink and Wullimann, 2001) or, more recently, in fact homologous to the ventral tegmental area (Luo et al., 2008). We report here that AR, ERα, ERβ, and PR are within all of these regions in *A. burtoni*, suggesting that steroid hormones may play an important role in modulating these neural systems involved in evaluating the salience of social and other stimuli.

CONCLUSIONS

We have demonstrated that AR, ERα, ERβ, and PR are expressed in brain regions that putatively regulate social behavior and evaluation of stimulus salience in a teleost brain. These findings suggest that steroid hormones may play an important role in the regulation of complex social behavior, behavioral plasticity, and the evaluation of a rewarding stimulus in *A. burtoni*. Building on this foundation, future work will use quantitative detection methods and pharmacological manipulations to further dissect the role steroid hormone receptors may play in regulating the remarkable plasticity of social behavior in this species either on their own or in concert with neuropeptide pathways and aminergic modulation.

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