

## Neural distribution of the nuclear progesterone receptor in the túngara frog, *Physalaemus pustulosus*<sup>☆</sup>

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### ABSTRACT

The gonadal steroid hormone progesterone plays an important role across all vertebrates in mediating female reproductive physiology and behavior. Many effects of progesterone are mediated by a nuclear progesterone receptor (PR), which is crucial for integration of external signals and internal physiological cues in the brain to produce an appropriate behavioral output. The túngara frog, *Physalaemus pustulosus*, is an excellent model system for the study of mechanisms by which sensory signals, such as auditory communication, are processed within neural circuits where mate choice decisions are made. To establish a framework for studying the neural basis of mate choice and social behavior in this species, we first describe the cytoarchitecture of the brain using Nissl-stained sections. Then, in order to better understand where progesterone acts to regulate social decisions, we determined the distribution of PR protein throughout the brain of *P. pustulosus* by immunohistochemistry. We found PR immunoreactivity in key brain regions known to modulate the processing of auditory cues and social behavior in other vertebrates. Due to its widespread distribution, PR likely also plays important roles in non-limbic brain regions that mediate non-social information processing. Further, we have colocalized PR with tyrosine hydroxylase, providing a functional context for the role of progesterone in mediating motivation and motor behavior. Our results significantly extend our understanding of hormonal modulation in the anuran brain and support the important role of the nuclear progesterone receptor in modulating female mate choice and receptivity in amphibians and across vertebrates.

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### 1. Introduction

Individuals integrate external cues through sensory systems, and these environmental signals can have both immediate and long-term effects on brain processes and behavior. One key channel for affecting such long-term changes is the modulation of gene expression (Morgan and Curran, 1989, 1991; Clayton, 2000;

Hofmann, 2003, 2010; Aubin-Horth and Renn, 2009). Social decision-making requires an integration of external and internal cues in the brain where information is processed and behavioral decisions are implemented by dedicated brain circuits. Sex steroid hormones can alter neural circuit function and properties (Ball and Balthazart, 2004; Beach, 1948; Lehrman, 1965). Since (classical) steroid hormone receptors act as transcription regulators, these

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**Abbreviations:** A, anterior thalamic nucleus; AA, anterior amygdaloid area; Acc, nucleus accumbens; Ad, anterodorsal tegmental nucleus; AH, anterior hypothalamus; aob, accessory olfactory bulb; Av, anteroventral tegmental nucleus; BST, bed nucleus of the stria terminalis; C, central thalamic nucleus; Cb, cerebellum; CeA, central amygdala; DB, diagonal band of Broca; DH, dorsal hypothalamic nucleus; Dp, dorsal pallium; DP, dorsal pallidum; e, postolfactory eminence; Ep, posterior entopeduncular nucleus; Gc, griseum centrale rhombencephali; gl, glomerular layer of the olfactory bulb; gr, granule cell layer of the olfactory bulb; Hv, ventral habenula; La, lateral thalamic nucleus, anterior division; LA, lateral amygdale; LH, lateral hypothalamic nucleus; Lp, lateral pallium; Lpd, lateral thalamic nucleus, posterodorsale; Lpv, lateral thalamic nucleus, posteroventrale; Ls, lateral septum; M, dorsal midline; MeA, medial amygdale; Mgd, magnocellular preoptic nucleus, dorsal part; Mgv, magnocellular preoptic nucleus, ventral part; ml, mitral cell layer of the olfactory bulb; Mp, medial pallium; Ms, medial septum; ON, optic nerve; Npv, nucleus of the periventricular organ; P, posterior thalamic nucleus; Pd, nucleus posterodorsalis tegmenti; POa, anterior preoptic area; Pv, nucleus posteroventralis tegmenti; Rm, nucleus reticularis medius; Rs, nucleus reticularis superior; SC, suprachiasmatic nucleus; Str, Striatum; Tect, optic tectum; Tel, telencephalon; Tor-L, torus semicircularis, laminar nucleus; Tor-P, torus semicircularis, principal nucleus; Tor-V, torus semicircularis, ventral area; TP, posterior tuberculum; Vd, descending trigeminal tract; VH, ventral hypothalamic nucleus; VLd, ventrolateral thalamic nucleus, dorsal part; VLv, ventrolateral thalamic nucleus, ventral part; Vm, nucleus motorius nervi trigemini; VM, ventromedial thalamic nucleus; VP, ventral pallidum.

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pathways are good candidates for integrating external signals into gene expression changes. An animal's hormonal state can mediate the integration of external cues and the way auditory signals are perceived. For example, in females of the plainfin midshipman fish, *Porichthys notatus*, hormonal state affects auditory sensitivity to male vocalizations (Sisneros et al., 2004). Gonadal steroid hormones can also have rapid non-genomic effects on behavior (Remage-Healey and Bass, 2006; Mani et al., 2009).

The effects of progesterone can be mediated by genomic and non-genomic mechanisms. Effects on gene transcription are transduced by the nuclear progesterone receptor (PR), and thus the characteristics of PR action arise from its specificity to its ligand and the DNA response element as well as its spatial and temporal pattern of expression. Importantly, besides a recently characterized conventional G protein-coupled progesterone receptor (Thomas, 2008; Mani et al., 2009), PR itself can also mediate non-genomic effects of progesterone, when it participates in a phosphorylation signal-transduction cascade (Zhu et al., 2008). In the context of behavior, the best characterized non-genomic interaction is between dopamine receptors and PR in facilitating female receptivity in rats (Mani et al., 2000; Frye, 2001). More generally, progesterone has been found to regulate diverse social behavior patterns in many vertebrates species, such as male and female sexual behavior, parental behavior, addiction, and aggression (Schneider et al., 2003; Crews, 2005; Wagner, 2006; Frye, 2007; Kabelik et al., 2008).

In amphibians, progesterone appears to influence female mate choice and receptivity, which is the best-studied social decision-making behavior in this vertebrate group. In female anurans, including túngara frogs, *Physalaemus pustulosus*, plasma progesterone levels are much higher during amplexus (Harvey et al., 1997; Itoh and Ishii, 1990), when females display the maximum frequency of reproductive behavior (Lynch and Wilczynski, 2005). Both estradiol and progesterone are required for receptive behavior in the clawed frog, *Xenopus laevis*, although receptivity did not increase with either hormone alone (Kelley, 1982). In the American toad, co-injection of progesterone and prostaglandin increases female receptivity to male mating calls as measured by the intensity and duration of phonotaxis, although treatment of prostaglandins alone will not elicit this behavior (Schmidt, 1985). Although recent work in female túngara frogs has shown that progesterone is not necessary for phonotaxis movement (Chakraborty and Burmeister, 2009), its role in receptivity or the mate choice process itself remains to be investigated. Since progesterone likely plays an important role in amphibian reproduction, it is surprising that the neural distribution of the progesterone receptor in this group is unknown. This information would give us a better understanding of which brain regions may be sites of modulation of social behavior by progesterone, especially in the light of recent insights into the neural circuitry underlying mate choice and female receptivity in the túngara frog (Hoke et al., 2004, 2005, 2007, 2008; Burmeister et al., 2008).

The túngara frog is an excellent model system to study the mechanisms by which sensory cues are transduced into molecular events within the neural circuits that govern behavioral decisions, such as mate choice. As in most anurans, túngara males produce species-specific advertisement calls that females use for species recognition and assessment of male quality (Ryan, 1985). Females will respond to both natural and synthetic calls in phonotaxis experiments, exhibiting a robust and repeatable approach towards broadcast calls, a behavior that is an unequivocal indication of mating call preference (e.g. Ryan and Rand, 1995; Phelps et al., 2006). Importantly, the anuran auditory system is biased towards detection and perception of conspecific mating calls (Wilczynski and Capranica, 1984), and details of these processes in túngara frogs have been revealed through studies of electrophysiology (Ryan et al., 1990; Wilczynski et al., 2001) and analysis of

immediate early gene expression (Hoke et al., 2004, 2005, 2007, 2008; Burmeister et al., 2008).

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. First, many studies indicate that the mesolimbic reward system (including the mid-brain dopaminergic system) is the neural network where evaluations of stimulus salience take place (Deco and Rolls, 2005; Wickens et al., 2007). Second, the neural substrates underlying social behavior, including female sexual behavior, have been proposed by Newman (1999) to form a “social behavior network”, mostly based on work in mammals. The core nodes of this network are involved in multiple forms of social behavior, are reciprocally connected, and – by definition – contain sex steroid hormone receptors. This framework has since been expanded to reptiles, birds, and teleosts (Newman, 1999; Crews, 2003; Goodson, 2005; O'Connell and Hofmann, 2011), yet has not been specifically applied to amphibians, although the involvement of several hypothalamic nodes of Newman's network has been discussed by Hoke et al. (2005). While the brain regions involved in the dopaminergic reward system and Newman's social behavior network are well studied in mammals, and increasingly in other amniotes, determining the homologs of these brain areas in the amphibian brain has been a challenge, especially for forebrain regions in the basal nuclei (Bruce and Braford, 2009; Marín et al., 1998). However, a consensus is emerging from neurochemical, hodological, and developmental studies that provide support for putative homologies for most of the relevant areas in the amphibian brain (Endepols et al., 2000; Marín et al., 1998; Smeets et al., 2000; Bruce and Braford, 2009; O'Connell and Hofmann, in press). These two neural networks can be used as a useful framework for understanding the neural underpinnings of female mate-choice and social decision-making in amphibians and in other vertebrates.

The main aim of this study is to test the hypothesis that PR is expressed in fore- and midbrain regions important for the regulation of social behavior and evaluation of stimulus salience. Towards this aim, we determined the distribution of PR in the female túngara frog brain, as progesterone plays an important role in female receptivity and mate choice in many vertebrate species. We also describe the basic architecture of the túngara frog brain, as no cytoarchitectonic description exists despite the importance of this model system for the study of female mate choice and sexual selection (Ryan, 2010). Together, a better understanding of the basic morphology of the túngara frog brain and the distribution of PR will facilitate functional studies directly related to the neural basis of mate choice and auditory communication. Finally, we also colocalize PR with tyrosine hydroxylase, in order to lay a foundation for functional studies into the interaction of PR and dopaminergic systems in the anuran brain.

## 2. Materials and methods

### 2.1. Animals

The animals chosen for this study were females housed in a breeding colony. The frogs were descendants of animals collected in Panama and maintained in 19-l aquaria or larger landscaping ponds that were converted to terraria. Frogs were maintained at 25 °C on a diet of crickets and wingless fruit flies, a 12:12 light cycle, and misted several days a week to maintain moisture and humidity levels similar to their native habitat.

We adopted the neuroanatomical nomenclature of Marín et al. (1998) for basal nuclei, Northcutt and Klitner (1980) for the telencephalon, Neary and Northcutt (1983) for the diencephalon, Wilczynski (1988) for the divisions of the torus (as originally described by Potter, 1965), and Gonzalez and Smeets (1994) for the hindbrain. All work was carried out in compliance with the Institutional Animal Care and Use Committee at The University of Texas at Austin.

### 2.2. Cresyl violet staining for cytoarchitecture

Túngara females ( $n = 5$ ) were sacrificed and the brain and skull were rapidly dissected and incubated in 4% formaldehyde in 1 × phosphate-buffered saline (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 OCT and storing at –80 °C. Brains were then sliced on a cryostat at 14 μm and thaw-mounted onto Super-Frost Plus slides (Erie Scientific Co., Portsmouth, NH) in four series that were stored at –80 °C for four to six weeks.

One series was used for cresyl violet (Nissl) staining. This series was warmed to room temperature and dehydrated in a desiccator for 1 h. Slides were then processed as follows: two incubations in water for 3 min each, followed by 15 min in cresyl violet staining solution (0.01% cresyl violet in water). Slides were then dipped in water and dehydrated in a series of ethanol and ending in xylene, and cover-slipped with Permount (Fisher Scientific, Itasca, IL).

### 2.3. Immunohistochemistry (IHC)

One series of brain sections was removed from –80 °C and air-dried before being fixed in chilled 4% formaldehyde in 1 × PBS, pH 7.4, for 10 min. Sections were then rinsed in PBS, and incubated in 3% hydrogen peroxide in PBS for 20 min. 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 2 min, the boiling citrate buffer was replaced two times and incubated for 5 min each, followed by a PBS wash. After blocking for 1 h in blocking solution (5% normal goat serum and 0.3% TritonX-100 in PBS), sections were incubated in primary antibody (PR 1:500, abcam 2767, monoclonal antibody raised against chicken PR) in PBS with 2% normal goat serum and 0.3% Triton-X-100 at room temperature overnight. Sections for PR colocalization with TH were incubated overnight in a mix of 1:500 anti-PR and 1:500 rabbit anti-TH (Millipore AB152). The specificity of the TH antibody to túngara antigen has been described in O'Connell et al. (2010).

Visualization with brightfield: sections were rinsed, incubated for 2 h in a biotinylated goat anti-mouse secondary antibody (1:200, Vector Laboratories), 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) as the substrate (Vector Labs). Finally, sections were counterstained with methylene green, dehydrated in an alcohol series and cover-slipped with Permount (Fisher Scientific, Itasca, IL). Control sections for the secondary antibody were processed with the same procedure except that primary antibody was omitted.

Visualization with fluorescence: sections were rinsed in 1 × PBS two times for 10 min and then incubated for 2 h in a mix of 1:200 Alexa Fluor 488 goat anti-mouse (Invitrogen A-21042) and 1:500 Texas Red goat anti-rabbit (Invitrogen T-2767) in 2% normal goat serum and 0.3% Triton-X 100 in 1 × PBS. Sections were then washed in 1 × PBS and then coverslipped in Vectashield Hardset Mounting Medium with DAPI (Vector Laboratories, H1500). Controls included slides that omitted primary antibody.

### 2.4. Western blot

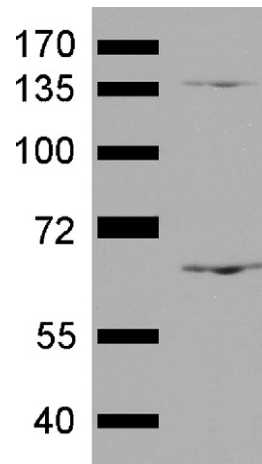
In order to determine whether the PR antibody would bind specifically to either one or both of the two PR subtypes in this frog species, we extracted protein from whole brain using a Mammalian Cell Lysis kit (Sigma) according to the manufacturer's instructions. Whole brain protein extract was run on an 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% TritonX-100, 0.1% Tween-20 in 1 × Tris-buffered saline (TBS)), incubated in primary antibody (1:2000 PR in 1 × TBS and 2% Na<sub>3</sub>B) for 1 h, washed five times for 3 min each in wash buffer, and then incubated in goat-anti-mouse HRP-conjugated antibody (Southern Biotech) in blocking solution for 30 min. After washing five times for 3 min each with wash buffer, the membrane was exposed to HRP substrate (Immobilon Western, Millipore) and exposed to film for 5 min. Using the PR antibody, two bands were visualized representing the putative PR-A and PR-B receptor proteins at the predicted sizes of 72 kD and 135 kD, respectively (Fig. 1). Two PR isoforms have been described in *X. laevis* (XPR-1, Genbank accession number AF279335, Tian et al., 2000; XPR-2, Genbank accession number AY007198, Bayaa et al., 2000) at similar molecular weights.

### 2.5. Photomicroscopy

Brightfield optics were used to visualize cresyl violet and immunohistochemical PR staining throughout the brain at low (5×) and high magnification (10×). Photographs were taken with a digital camera (AxioCam MRc, Zeiss) attached to an 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.

Fluorescence signal was detected using a Zeiss AxioImager.A1 AX10 microscope equipped with DAPI, FITC, and Texas Red filters. Photographs were taken in each of the DAPI FITC, and Texas Red channels, imported into Adobe Photoshop CS3 and assembled into merged images.



**Fig. 1.** Confirmation of PR antibody specificity. Western blot was used to confirm specificity of the PR antibody against *P. pustulosus* whole brain extract. Note that two bands were visualized representing the putative PR-A and PR-B receptor proteins at the predicted sizes of 72 kD and 135 kD, respectively. Ladder units are in kDa.

## 3. Results

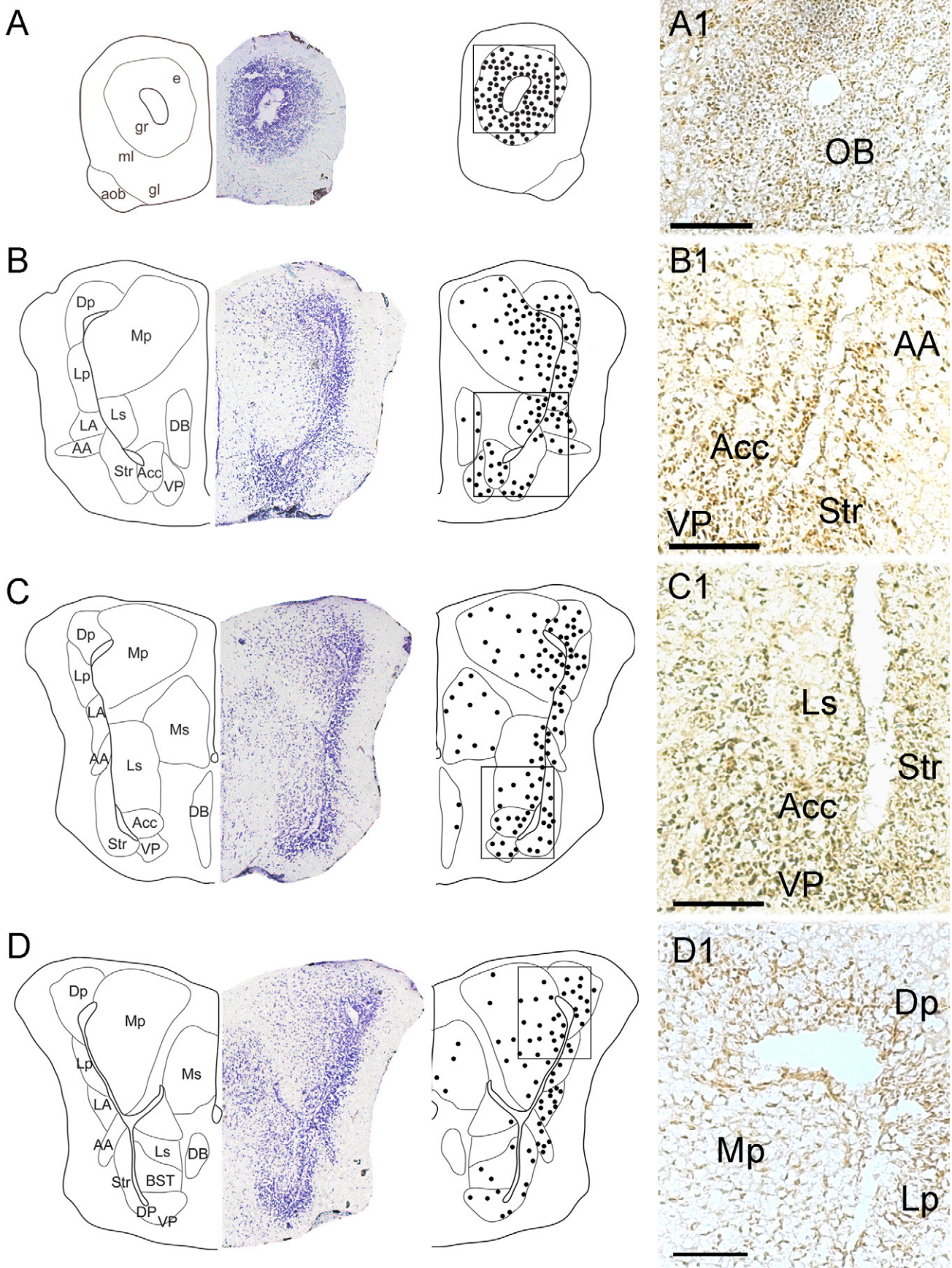
Here we describe the cytoarchitecture and distribution of PR in the forebrain, midbrain, and hindbrain of the female túngara frog (Figs. 2–4). For each representative section of the map, the nomenclature is displayed on the left side while a micrograph of the cresyl violet staining is shown on the right. PR-immunoreactive cells are found widely distributed throughout the brain of female *P. pustulosus*. PR-immunoreactivity was observed as both cytosolic and nuclear. This antibody recognized both isoforms of PR (Fig. 1), and therefore the distribution presented here reflects both, although we cannot account for instances where only one isoform is expressed in a particular brain region. In the following, we present a distribution map along with photomicrographs of representative brain areas containing PR protein (Figs. 2–4). For each representative section of the map, protein staining by immunohistochemistry is represented by one dot per 10 immunoreactive cells. Control slides omitting the PR antibody showed no staining.

### 3.1. Forebrain

The accessory olfactory bulb is located on the lateral-ventral region of the main olfactory bulb (Fig. 2A). The main olfactory bulb is made up of several cell layers: the glomerular layer (gl), mitral layer (ml), granule layer (gr) and the postolfactory eminence (e). PR is present only within the postolfactory eminence and the granule cell layer but not the mitral or glomerular layer (Fig. 2A1).

Caudal to the olfactory bulb, the pallium is organized into three subregions: medial (Mp), dorsal (Dp), and lateral (Lp). The medial pallium lies on the dorsomedial wall, while the dorsal and lateral pallium hug the dorsolateral wall of the ventricle and contain cells that are much more compact compared to the dispersed cells of Mp. PR immunoreactivity is present within all three pallial regions (Fig. 2D1).

The subpallium is arranged along the ventral portion of the ventricle. The amygdaloid complex lies on the ventrolateral wall of the ventricle and is composed of the lateral amygdala (LA), anterior amygdaloid (AA) complex, medial amygdala (MeA), and central amygdala (CeA). PR is present within each of these regions of the amygdaloid complex (Fig. 2B). Ventral to the amygdaloid complex is the striatum (Str). Medial to the striatum is the nucleus accumbens (Acc), whose homology with the mammalian nucleus



**Fig. 2.** Distribution of progesterone receptor protein in the forebrain. The first column of representative brain sections shows a single hemisphere in the transverse plane stained with cresyl violet on the right and line drawings representing cell groups on the left. PR protein distribution is depicted in the second column with PR protein represented by dots, and each dot represents 10 cells positive for PR. The micrograph in the top row shows PR protein (A1) in the olfactory bulb (OB). The micrograph in the

accumbens is based upon neurochemical and hodological evidence (Gonzalez and Smeets, 1994). One of the few cell groups not along the ventricle wall is the diagonal band of Broca (DB), which runs vertically along the midline. Medial to the nucleus accumbens is the pallidum, which more rostrally is only composed of the ventral portion, but more caudally is divided into dorsal and ventral subregions (DP and VP, respectively; Fig. 2B–D). PR is abundant within these basal nuclei regions including the striatum, nucleus accumbens and the ventral pallidum.

The septal nuclei make up the dorsomedial subpallium and are divided into lateral and medial regions (Ls and Ms, respectively). PR is present within both the lateral and, more sparse, the medial septum. More caudally, as cell density in the medial septum increases, the rostral portion of the bed nucleus of the stria terminalis (BST) becomes visible on the medial wall of the lateral ventricle. Cell density in the BST is low compared with the lateral septum and lower yet compared with the dense cell cluster of the pallidum. There is PR present within the rostral portion of the BST, with the number of PR immunoreactive cells increasing caudally (Figs. 2D and 3A).

As the anterior preoptic nucleus (POa) begins to emerge around the third ventricle (Fig. 3A), the BST is stretched above the anterior commissure and contains PR protein. As the third ventricle expands, the POa extends more dorsally and the posterodorsal and posteroventral magnocellular preoptic nucleus (Mgd and Mgv, respectively) emerge (Fig. 3B). Regions of the preoptic area including the dorsal and ventral magnocellular preoptic nucleus and the POa, contain an abundance of PR protein (Fig. 3A1 and B1).

Caudal to the preoptic nuclei, the ventromedial thalamic nucleus (VM) emerges as the rostral portion of the thalamic nuclei become visible. Dorsal to the VM is the ventral habenula (Hv). The ventromedial thalamic nucleus and the ventral habenula also have PR protein (Fig. 3B). More caudally, the dorsal diencephalon is dominated by thalamic nuclei (Fig. 3C). Lateral to VM are the dorsal and ventral regions of the ventrolateral thalamic nucleus (VLd and VLv, respectively). Dorsal to these thalamic nuclei is a very large cell group that composes the anterior thalamic nucleus (A). The dorsal and ventral regions of the ventrolateral thalamic nucleus (VLd and VLv) and the anterior thalamic nucleus also contain PR-immunoreactive cells (Fig. 3C). Ventral to these thalamic regions, the hypothalamic regions become distinct. The anterior hypothalamus (AH) lays medial against the third ventricle wall. There is an abundance of PR-immunoreactivity in the anterior hypothalamus (Fig. 3C1). Caudal to the anterior hypothalamus, the ventral hypothalamic nucleus (VH) emerges as the cell group surrounding the third ventricle (Fig. 3D). The lateral hypothalamic nucleus (LH) is lateral to ventral hypothalamic nucleus and comparatively has much less cells. PR-immunoreactivity is abundant in the ventral hypothalamic nucleus, but not seen in the lateral hypothalamus (Fig. 3D1).

The suprachiasmatic nucleus (SC) appears dorsally where these cells are immediately ventral to VM and contain PR protein. Lateral to suprachiasmatic nucleus the posterior entopeduncular nucleus (Ep) appears as a group of cells in a curved shape. Dorsal to VM, the central (C) and lateral regions of the thalamic nucleus become apparent. The anterior region of the lateral thalamic nucleus (La) appears more rostral while the posterodorsal and posteroventral regions (Lpd and Lpv, respectively) are more caudal. These latter two subregions of the lateral thalamic nucleus are lateral to the posterior thalamic nucleus (P), a very large grouping of cells close

to the midline (Fig. 3E). All of these thalamic nuclei contain PR protein.

Ventral to the large grouping of thalamic nuclei is the posterior tuberculum (TP), and ventral to that are the dorsal and ventral hypothalamic nuclei (DH and VH) along the fourth ventricle, each of which contains PR protein (Fig. 3E1). Medial to DH and ventral to TP is the small nucleus of the periventricular organ (NPv), which also contains PR-immunoreactivity.

### 3.2. Midbrain and hindbrain

The midbrain and hindbrain contain many regions involved in motor control (Fig. 4). PR is also present in the caudal midbrain and hindbrain, although the distribution is sparser than in the forebrain. The optic tectum (Tect) contains many cell layers that are characteristic of this region in many vertebrates and contains PR protein (Fig. 4A). Ventral to the Tect, the torus semicircularis (Tor) can be divided into three distinct clusters (Wilczynski, 1988; Wilczynski and Endepols, 2007). Interestingly, the laminar and magnocellular nuclei of the torus semicircularis contain abundant PR protein, whereas the principal nucleus of the torus semicircularis shows little PR-immunoreactivity (Fig. 4A).

Ventral to Tor are the anterodorsal and anteroventral tegmental nuclei (Ad and Av, respectively), both of which contain PR protein. The tegmental nuclei continue more caudally to form the tegmentum posteriodorsale and posteroventrale (Pd and Pv, respectively; Fig. 4B) and also contain PR protein. Ventral to Pv, the nucleus reticularis superior (Rs) appears, which contains PR protein and generally has many more cells than Pv. More caudally, as the cerebellum emerges, Rs gives way to the nucleus reticularis medialis (Rm) that contains an abundance of PR protein (Fig. 4C1). Dorsal to Rm, the nucleus motorius nervi trigemini (Vm) appears, and dorsolateral to Vm is the descending trigeminal tract (Vd). The descending trigeminal tract (Vd) and the nucleus motorius nervi trigemini (Vm) both contain PR-immunoreactive cells. Next, a distinct cell group ventromedial to Rm forms the griseum centrale rhombencephali (Gc), which has an abundance of PR protein. Finally, there are PR-sparse immunoreactive cells within the cerebellum.

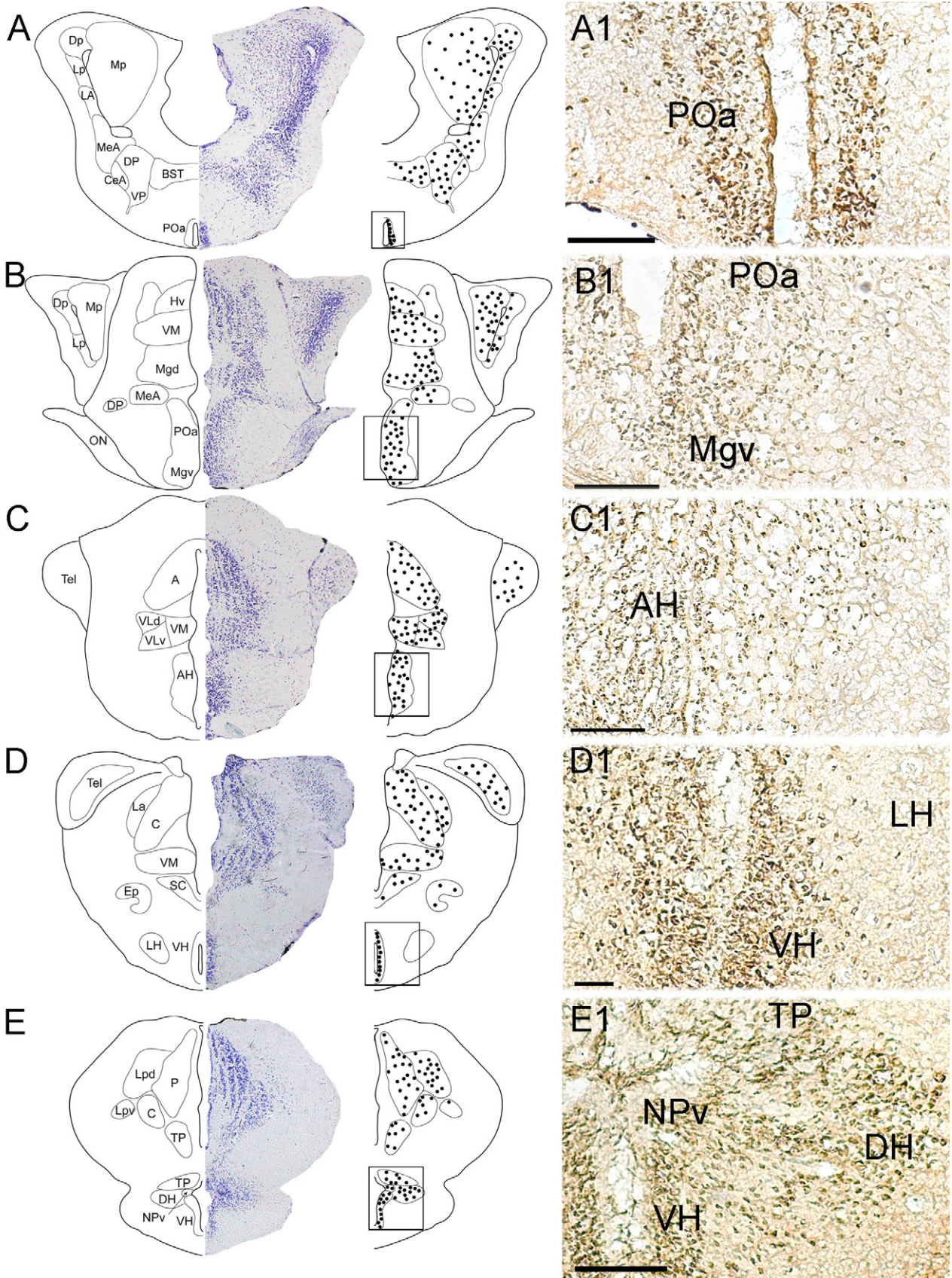
### 3.3. Localization of PR in dopaminergic cells

To evaluate the functional implications of the distribution of PR cells in the túngara frog brain, we asked whether is PR colocalized with TH. We found PR to be co-localized with putative dopaminergic cells in the posterior tuberculum (Fig. 5B). Importantly, PR is not expressed in all cells within this region, as can be seen when comparing PR-immunoreactivity to the DAPI counterstain, suggesting that PR may be playing selective roles in these brain regions.

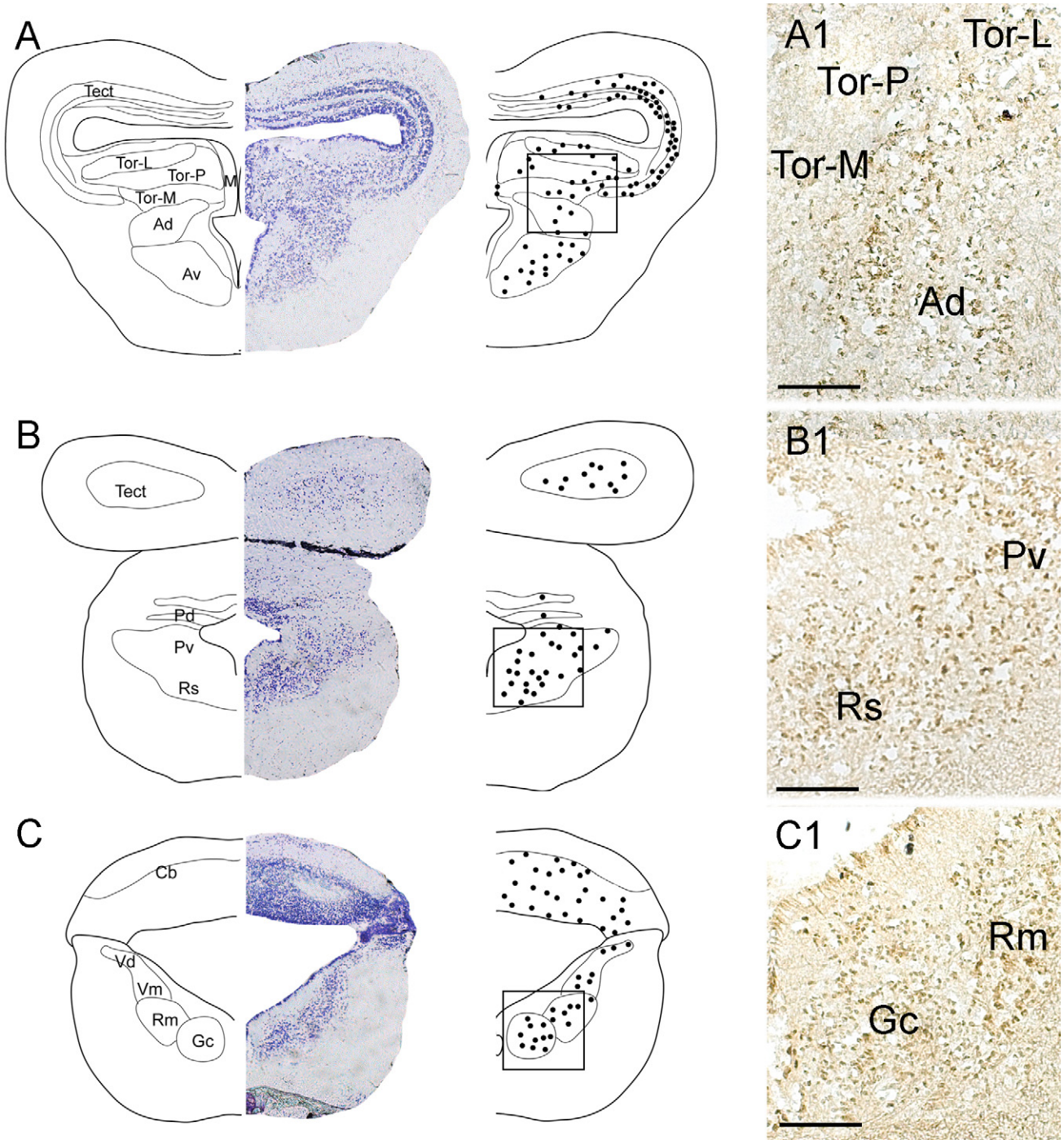
## 4. Discussion

We have provided here the first complete description of the distribution of PR in the túngara frog brain as well as the cytoarchitecture of these neotropical anurans. PR is widely distributed throughout the brain of this amphibian, thereby elucidating which regions of the brain are possible targets of progesterone modulation. We find PR in brain regions that are known across vertebrates to modulate social behavior and/or encode stimulus salience, as expected. However the unexpected

second row shows PR protein (B1) in the nucleus accumbens (Acc), striatum (Str), ventral pallidum (VP), and anterior amygdaloid area (AA). The third panel contains a micrograph showing PR protein (C1) patterns in the Acc, VP, Str, and lateral septum (Ls). The fourth panel contains a micrograph showing PR protein (D1) in the dorsal, lateral, and medial pallidum (Dp, Lp, and Mp, respectively). All scale bars are shown at 100  $\mu$ m.



**Fig. 3.** Distribution of progesterone receptor protein in the diencephalon. The first column of representative brain sections shows a single hemisphere in the transverse plane stained with cresyl violet on the right and line drawings representing cell groups on the left. PR protein distribution is shown in the second column with PR protein represented by dots, and each dot represents 10 cells positive for PR. The micrograph in the top row shows PR protein (A1) in the anterior preoptic area (POa). The micrograph in the second row shows PR protein (B1) in the POa and ventral magnocellular preoptic nucleus (Mgv). The third panel contains a micrograph showing PR protein (C1) patterns



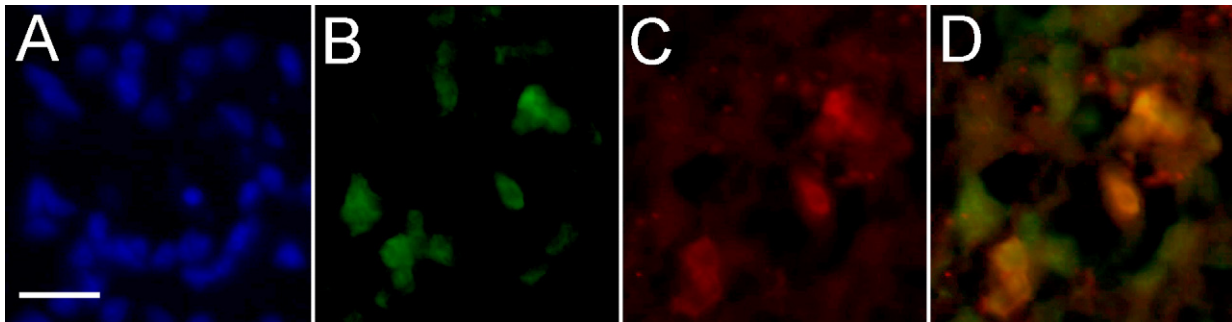
**Fig. 4.** Distribution of progesterone receptor protein in the midbrain and hindbrain. The first column of representative brain sections shows a single hemisphere in the transverse plane stained with cresyl violet on the right and line drawings representing cell groups on the left. PR protein distribution is depicted in the second column with PR protein represented by dots, and each dot represents 10 cells positive for PR. The micrograph in the top row shows PR protein (A1) in the torus semicircularis (Tor) and the anterodorsal tegmental nucleus (Ad). The micrograph in the second row shows PR protein (B1) in the nucleus posteroventralis tegmenti (Pv) and nucleus reticularis superior (Rs). The third panel contains a micrograph showing PR protein (C1) patterns in the nucleus reticularis medius (Rm) and griseum centrale rhombencephali (Gc). All scale bars are shown at 100  $\mu\text{m}$ .

widespread distribution suggests other roles for PR than that of social behavior. Further, we have colocalized PR with TH in the posterior tuberculum, providing a functional framework in which to study the role of progesterone in mediating behavior in concert with dopaminergic systems.

#### 4.1. Comparison of cytoarchitecture to other amphibians

The brain organization of amphibians has been of interest to comparative neurobiologists for many decades in search of the neural adaptations that took place in the anamniote–amniote

in the anterior hypothalamus (AH) and suprachiasmatic nucleus (SC). The fourth panel contains a micrograph showing PR protein (D1) in ventral hypothalamic nucleus (VH). The fifth panel contains a micrograph showing PR protein (E1) patterns in the VH, dorsal hypothalamic nucleus (DH), posterior tuberculum (TP), and nucleus of the periventricular organ (NPv). All scale bars are shown at 100  $\mu\text{m}$ .



**Fig. 5.** Co-localization of PR with tyrosine hydroxylase in putative dopaminergic cells in the posterior tuberculum. Panel A shows the DAPI channel, panel B shows PR in the FITC channel, panel C shows TH in the Texas-Red channel and panel 4 shows the merged image of the FITC and Texas-Red channels. Scale bar is at 20  $\mu\text{m}$ .

transition, as vertebrates began occupying terrestrial habitats. We have described the cytoarchitecture of the túngara frog in order to facilitate future studies investigating the neural basis of auditory communication and female mate choice, for which this species has become an attractive model system (Ryan, 2010). Although there may be quantitative differences in cell number and regional volume, the regional organization of the anuran brain is remarkably uniform (Northcutt and Kicliter, 1980; Frontera, 1952). In anurans an important step in auditory processing takes place in the inner ear, as the peak sensitivity of the two inner ear organs, the amphibian papilla and the basilar papilla, match peaks of spectral energy in the species' mating call (Wilczynski and Capranica, 1984; Ryan, 1986; Gerhardt and Schwartz, 2001). Nevertheless, substantial processing takes place throughout the auditory system, especially in the large auditory nucleus in the mid-brain, the torus semicircularis (Tor) (Wilczynski and Capranica, 1984; Feng et al., 1990). Central neural pathways in the túngara frog that are implicated in auditory processing of mating calls (Hoke et al., 2004), social decision making (Hoke et al., 2005), integration of sensory input and motor output (Hoke et al., 2007), and sexual differences in male and female response to stimulus variation (Hoke et al., 2008) have all been identified in túngara frogs using expression of immediate early genes as markers for neural activation. This cytoarchitecture information, when considered with immediate early gene expression, provides a strong foundation for future molecular studies into the neural basis of decision-making in this species.

#### 4.2. Comparison of progesterone receptor distribution in amphibians

The putative distribution of PR in amphibians has been studied previously in *X. laevis* by autoradiography using  $^3\text{H}$ -R5020 (Roy et al., 1986). In that study, progesterone target cells were found in the ventrolateral striatum, ventral septum, preoptic area, amygdala, and the laminar nucleus of the torus semicircularis. We have found PR protein in all these regions with the exception of the ventral septum. However, this discrepancy is difficult to reconcile, as Roy and colleagues showed only schematic representations of horizontal sections. Other discrepancies between regions containing PR in *P. pustulosus* and the lack of progesterone target-cells in *X. laevis* – as seen in the nucleus accumbens, posterior tuberculum, and pallial nuclei, and many other regions – could be due to sensitivity of the autoradiography or species differences. More studies examining the distribution of PR across amphibians would give us a better understanding of the putative neural sites of progesterone action.

#### 4.3. Comparison of progesterone receptor distribution to other vertebrates

In the following we compare the distribution of the progesterone receptor in the túngara frog to other vertebrates (Table 1). Although our discussion here focuses on two candidate neural networks that appear to regulate social decision-making in vertebrates, Newman's social behavior network (Newman, 1999) and the mesolimbic dopamine system, the wide distribution of PR

**Table 1**  
Comparison of PR distribution across vertebrates.

Brain region	Frog	Teleost	Reptile	Bird	Mammal
Olfactory bulb	+	+	+	?	–
Nucleus accumbens	+	+	+	+	+
Striatum	+	+	+	+	+
Lateral septum	+	+	+	+	+
Hippocampus	+	+	+	+	+
Amygdala	+	+	+	–	+
Bed nucleus of the stria terminalis	+	+	+	+	+
Preoptic area	+	+	+	+	+
Thalamus	+	+	+	+	+
Lateral hypothalamus	–	+	+	?	+
Ventral hypothalamus	+	+	+	+	+
Torus semicircularis	+	+	+	?	?
Ventral tegmental area	+	+	+	–	+
Central grey	+	+	?	–	+

**Legend:** Distribution of PR in homologous brain regions across vertebrates. Overall, the distribution of PR is widespread. References: Frog: *P. pustulosus*, present study; teleost: *A. burtoni* (Munchrath and Hofmann, 2010), *D. rerio* (Hanna et al., 2010); reptile: *C. inornatus* and *C. uniparens* (O'Connell et al., 2011); bird: *T. guttata* (Lubischer and Arnold, 1990), *G. domesticus* (Sterling et al., 1987), songbirds (Gahr, 2001); Mammal: *R. norvegicus* (Kato et al., 1994), *M. musculus* (Shughrue et al., 1992), *C. porcellus* (Warembourg et al., 1986).



in other brain regions suggests that progesterone likely modulates non-social behavior as well. While identifying amphibian homologies for the brain regions that are part of these systems has not always been straightforward, a consensus has been emerging (Endepols et al., 2000; Marín et al., 1998; Smeets et al., 2000; Bruce and Braford, 2009; O'Connell and Hofmann, in press).

The social behavior network was originally proposed for mammals (Newman, 1999) – and more recently applied to other vertebrate classes (Crews, 2003; Goodson, 2005; O'Connell and Hofmann, 2011) – and contains mostly hypothalamic regions that express steroid hormone receptors. The nodes of this network include the preoptic area, anterior hypothalamus, ventromedial hypothalamus, medial amygdala and bed nucleus of the stria terminalis (BST), periaqueductal grey/central grey, and the lateral septum. These regions contain steroid hormone receptors in every vertebrate class studied including reptiles (Young et al., 1994; O'Connell et al., 2011), teleosts (Hanna et al., 2010; Munchrath and Hofmann, 2010), birds (Askew et al., 1997; Gahr, 2001; Sterling et al., 1987), and mammals (Quadros et al., 2008; Lonstein and Blaustein, 2004; Kato et al., 1994). We have shown here that PR is expressed in each of these brain regions in *P. pustulosus*, providing neurochemical evidence in support of these amphibian homologies in the social behavior network, although further manipulative and behavioral studies are still necessary.

The other neural network of fundamental importance in the regulation of behavior, the mesolimbic reward system, centers around the dopaminergic ventral tegmental area, which projects to many forebrain nuclei and is important for reinforcing learned behavior (Young and Wang, 2004). Regions that receive input from this dopaminergic node include the basolateral amygdala, hippocampus, nucleus accumbens, ventral pallidum, striatum, BNST, and the lateral septum. Most of these brain nuclei contain the PR in reptiles (Young et al., 1994; O'Connell et al., 2011), teleosts (Hanna et al., 2010; Munchrath and Hofmann, 2010), birds (Askew et al., 1997; Gahr, 2001; Sterling et al., 1987), and mammals (Quadros et al., 2008; Lonstein and Blaustein, 2004; Kato et al., 1994). The putative amphibian homologies to these forebrain nuclei are more contentious than those of the social behavior network (Marín et al., 1998; Moreno and González, 2004; Brox et al., 2004; Moreno et al., 2004; Roth et al., 2007) and should still be considered tentative until more developmental, hodological, neurochemical, and lesion/stimulation studies are reported (for review see O'Connell and Hofmann, in press). The putative homologies are as follows: the medial pallium (Mp) as a putative homologue to the mammalian hippocampus (Roth and Westhoff, 1999), the ventral region of the lateral pallium (Lpv) as the putative homologue to the mammalian basolateral amygdala (Bruce and Braford, 2009), and the posterior tuberculum (TP) as a putative homolog to the mammalian ventral tegmental area/substantia nigra pars compacta (Smeets and Reiner, 1994). All other brain regions in the dopaminergic reward system in the amphibian brain are named similar to their putative mammalian homologues. We report here that PR is found within all of these regions in *P. pustulosus*, suggesting that progesterone may play important roles in modulating this neural system involved in evaluating the salience of social and other stimuli. Further, we have shown that PR co-localizes with putative dopaminergic cells in the TP. Previous work investigating immediate early gene expression in female túngara frogs has shown a higher response in the posterior tuberculum when exposed to conspecific male calls (Hoke et al., 2005). This result suggests that a cellular response in this region may promote female-typical behavior patterns in this species, although whether this activation involves PR action within dopaminergic cell groups remains to be seen.

#### 4.4. Possible role for progesterone in modulating the auditory pathway

The torus semicircularis (Tor) is the major center of integration auditory information in amphibians (Wilczynski and Capranica, 1984). The principal nucleus of Tor can be traced to the central and posterior thalamic nuclei (C and P, respectively; Feng and Lin, 1991; Matesz and Kulik, 1996; Luksch and Walkowiak, 1998), while the laminar and magnocellular nuclei of Tor project to di- and telencephalic areas (Wilczynski and Endepols, 2007). Auditory activity has also been recorded in anterior, lateral, and ventral thalamic nuclei (Mudry et al., 1977; Megela and Capranica, 1981). Each of these regions contain PR protein in the female túngara frog, and therefore progesterone could be modulating the processing of auditory inputs at any of these levels, although site-specific manipulations of PR in conjunction with electrophysiology would be needed to give a better understanding of this modulation.

The auditory pathway continues through the thalamic nuclei and projects to the striatum (Wilczynski and Northcutt, 1983), which is one of the two regions in the telencephalon that is responsive to auditory stimuli. The other region is the medial pallium (Mp; Mudry and Capranica, 1980), which is considered to be homologous to the mammalian hippocampus (Northcutt and Kicliter, 1980). PR protein has been found in both the striatum and Mp in this species and thus provides another avenue of progesterone modulation of auditory input.

#### 4.5. Functional implications of progesterone in modulating social decision-making

In female vertebrates, progesterone and other steroid hormones are important in the regulation of reproductive physiology and sexual behavior, including mate choice and receptivity. Interestingly, the effects of progesterone on these behavior patterns vary across vertebrate classes. Exogenous progesterone increases receptivity in mammals (White et al., 2007) and amphibians (Schmidt, 1985), but decreases female-typical behavior in reptiles (Godwin et al., 1996). Similarly, in canaries, injection of exogenous progesterone leads to a reduction in female responsiveness to male songs (Leboucher et al., 2000). Previous studies in female túngara frogs have shown progesterone is not necessary for phonotaxis (Chakraborty and Burmeister, 2009), but whether progesterone plays a role in female preference or receptivity remains to be determined.

The interaction of dopamine and progesterone has been well studied in mammals, where PR is required for both progesterone- and dopamine-facilitated lordosis (Mani et al., 1996). In fact, Mani et al. (2000) suggest that cross-talk between the progesterone and dopamine pathways is important for integration of signals that modulate female receptivity in rodents. These pathways may also be important for female mate choice in amphibians. Importantly, we have shown here that PR is expressed within dopaminergic cells in the TP, providing a functional framework for future studies in which to test the interaction of progesterone and dopamine in túngara frog mate choice.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchemneu.2011.01.002.

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