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Characterization of the Dopamine System in the Brain of the Túngara Frog, *Physalaemus pustulosus*

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Key Words

DARPP-32 · Dopamine receptor · Mate choice · Mesolimbic reward system · Posterior tuberculum · Tyrosine hydroxylase

Abstract

Dopamine is an evolutionarily ancient neurotransmitter that plays an essential role in mediating behavior. In vertebrates, dopamine is central to the mesolimbic reward system, a neural network concerned with the valuation of stimulus salience, and to the nigrostriatal motor system and hypothalamic nuclei involved in the regulation of locomotion and social behavior. In amphibians, dopaminergic neurons have been mapped out in several species, yet the distribution of dopaminoreceptive cells is unknown. The túngara frog, *Physalaemus pustulosus*, is an excellent model system for the study of neural mechanisms by which valuations of stimuli salience and social decisions are made, especially in the context of mate choice. In order to better understand where dopamine acts to regulate social decisions in this species, we have determined the distribution of putative dopaminergic cells (using tyrosine hydroxylase immunohistochemistry) and cells receptive to dopaminergic signaling (using DARPP-32 immunohistochemistry) throughout the brain of *P. pustulosus*. The distribution of dopaminergic cells was comparable to other anurans. DARPP-32 immunoreactivity was identified in key brain regions known to modulate social behavior in other vertebrates including the proposed anuran homologues of the mammalian amygdalar complex, nucleus accumbens, hippocampus, striatum, preoptic area, anterior hypothalamus, ventromedial hypothalamus, and ventral tegmental area/substantia nigra pars compacta. Due to its widespread distribution, DARPP-32 likely also plays many roles in non-limbic brain regions that mediate non-social information processing. These results significantly extend our understanding of the distribution of the dopaminergic system in the anuran brain and beyond.

Introduction

The brain processes that govern decision-making in complex social contexts are poorly understood. However, insight into the proximate mechanisms underlying the selection of behavioral responses to an external stimulus is of fundamental importance for explaining the evolution and survival value of these behavior patterns [Tinbergen, 1963]. There are 2 neural networks that have been implicated in behavioral decisions that either encode stimulus salience or regulate the expression of social be-

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Abbreviations used in this paper

A	anterior thalamic nucleus
AA	anterior amygdaloid area
Acc	nucleus accumbens
Ad	anterodorsal segmental nucleus
AH	anterior hypothalamus
aob	accessory olfactory bulb
Av	anteroventral segmental 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, posteriodorsal
Lpv	lateral thalamic nucleus, posterioventral
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	toris semicircularis, laminar nucleus
Tor-P	toris semicircularis, principal nucleus
Tor-V	toris 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

havior. The neural network where the salience of a stimulus is evaluated is the mesolimbic reward system (including, but not limited to the mid-brain dopaminergic system [Deco and Rolls, 2005; Wickens et al., 2007]). The neural circuitry that regulates social behavior was originally described in mammals as the 'social behavior network' [Newman, 1999], but has recently been expanded to reptiles, birds, and teleosts [Crews, 2003; Goodson, 2005]. The core nodes of Newman's network are reciprocally connected (mostly hypothalamic) regions that are – by definition – involved in multiple forms of social behavior and contain sex steroid hormone receptors. Despite their utility in research on other major vertebrate lineages, these frameworks have not yet been specifically applied to amphibians, although the involvement of several hypothalamic nodes in this network has been explored [Hoke et al., 2005].

The biogenic amine dopamine (DA) is an evolutionarily ancient molecule present in most eukaryotes. In all animals, DA plays an important role in modulating neural activity [Wintle and Van Tol, 2001; Callier et al., 2003; Kulma and Szopa et al., 2007]. In vertebrates, DA is important in the context of social behavior and decision-making, as this neurotransmitter plays a crucial role in encoding the rewarding properties of stimuli [Young and Wang, 2004; Wickens et al., 2007]. DA also plays an important role in modulating motor output through the nigrostriatal motor system [Mogenson et al., 1980; Girault and Greengard, 2004]. Although the role of the dopaminergic system with respect to social decision-making has been well characterized in mammals, and to a lesser extent in birds [Hara et al., 2007; Huang and Hessler, 2008; Heimovics and Riters, 2008], not much is known about the role of the dopaminergic system in modulating social decision-making in amphibians.

DA signaling reinforces incentive salience of many behavior patterns including aggression, sexual behavior, learning and memory [Young and Wang 2004; Hull and Dominguez, 2007; Kindt et al., 2007; Ryding et al., 2008; Dalley and Everitt, 2009; Krashes et al., 2009]. The rate-limiting enzyme in the DA synthesis pathway is tyrosine hydroxylase (TH), which is responsible for converting the amino acid L-tyrosine to L-DOPA [Levitt et al., 1965], and is regarded as a marker for dopaminergic cells. In dopaminoreceptive cells, DA stimulates phosphorylation of the phosphoprotein, dopamine and cAMP-regulated phosphoprotein-32 (DARPP-32) through a cascade of events involving the activation of the D_1-family dopamine receptors, subsequent increase in cAMP level, and activation of protein kinase A [Walaas and Greengard,

1984]. The presence of DARPP-32 in dopamine-sensitive neurons is necessary for signal transduction via D_1-agonists [Frye and Walf, 2010]. Thus, DARPP-32 has become a marker for cells receiving dopaminergic input through the D_1-like family of DA receptors.

Although much work has been done to understand the role of dopamine in locomotion and feeding behavior in amphibians [Glagow and Ewert, 1999; Endepols et al., 2004, Chu and Wilczynski, 2007], surprisingly few studies have examined the role of dopamine in reinforcement behavior in amphibians. Work in birds would suggest that in the context of mate selection, dopaminergic neurons within the female mesolimbic reward system might encode the salience of auditory signals emitted by males [Hara et al., 2007; Huang and Hessler, 2008; Heimovics and Riters, 2008]. Marín and colleagues [1995] have described the mesolimbic reward system in anurans as dopaminergic projections originating from the posterior tuberculum to the nucleus accumbens. Dopaminergic neurons in the posterior tuberculum are required for phonotaxis behavior in female grey tree frogs [Endepols et al., 2004] and vary in number with sex and gonadal hormone levels in Northern leopard frogs [Chu and Wilczynski, 2002; Wilczynski et al., 2003].

The túngara frog, *Physalaemus pustulosus*, is an excellent model system for the study of animal communication, female mate choice, and evolution of sexually dimorphic traits as a consequence of sexual selection [Ryan, 2010]. As in most anurans, túngara frog males produce species-specific advertisement calls that females use to choose conspecific mates and to further assess male quality [Ryan, 1985]. In phonotaxis experiments, females exhibit a robust approach to both natural and synthetic calls that is an unambiguous indicator of mate choice [e.g. Ryan and Rand, 1995; Phelps et al., 2006]. Mate preference by females is influenced by both internal physiological cues, such as hormone levels, and external signals, such as the complexity of the mating call [Ryan and Rand, 1995; Lynch and Wilcynski, 2006; Chakraborty and Burmeister, 2009]. The mechanistic basis of sensory integration has been well studied in túngara frogs with electrophysiology [Ryan et al., 1990; Wilczynski et al., 2001] and quantification of immediate early gene expression [Hoke et al., 2004, 2005, 2007a, b, 2008; Burmeister et al., 2008].

The characterization of the dopamine system in anamniote brains has increased our understanding of its evolution, as putative homologies with mammals have been proposed [González and Smeets, 1994; Rink and Wullimann, 2002]. The distribution of dopamine-producing cells in the amphibian brain in particular has been well studied in several species including the African clawed frog (*Xenopus laevis* [González et al., 1993]), the marsh frog (*Rana ridibunda* [González and Smeets, 1991]), the fire-bellied toad (*Bombina orientalis* [Endepols et al., 2006]), and the northern leopard frog (*Rana pipiens* [Wilczynski et al., 2003]). However, it has recently become apparent that all vertebrates except mammals have 2 paralogs encoding TH: *th1* and *th2* [Filippi et al., 2010; Yamamoto et al., 2010]. This finding likely opens up new avenues of research into the distribution and function of the 2 paralogs in the anuran brain, as this literature so far has relied exclusively on a monoclonal antibody specific to th1 only. Knowing where DA is produced is useful, but knowing where it acts is central to understanding its effect on behavior. However, to our knowledge, the distribution of the dopamine-receptive neurons has not been described throughout an amphibian brain, although the description of TH fibers may indicate brain regions that express dopamine receptors [González and Smeets, 1994]. Also, the distribution of D_2 receptors has been mapped out in the anuran midbrain of *X. laevis, Discoglossus pictus*, and *B. orientalis* [Endepols et al., 2000]. Characterization of the dopaminergic system in an amphibian would not only reveal which brain regions may be sites where the evaluation of incentive salience occurs, but would also give us a better understanding of putative homologies with other vertebrates.

In this study we aimed to first characterize the distribution of putative dopaminergic cells throughout an anuran brain using an antibody to TH. We also tested the hypothesis that cells receiving dopaminergic input are widely distributed throughout the brain of the túngara frog by using an antibody to DARPP-32, a signaling molecule generally regarded as a marker for dopaminoreceptive cells. We predicted that DARPP-32 would be present in brain regions known to be important for processing of auditory input and the regulation of social behavior in amphibians and higher vertebrates.

Materials and Methods

Animals

The animals chosen for this study were females from a breeding colony maintained at the University of Texas Austin. The frogs were descendants of animals collected in Panama and maintained in 19-liter 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

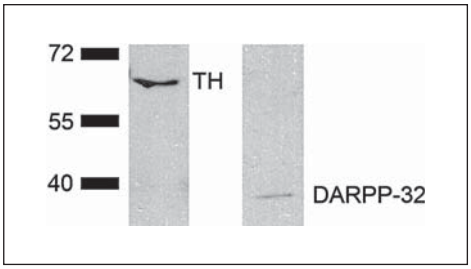


Fig. 1. Confirmation of antibody specificity. Western blot was used to confirm specificity of the TH and DARPP-32 antibodies against *P. pustulosus* whole brain extract. Ladder units are in kDa.

several days a week to maintain moisture and humidity levels similar to their native habitat [Romero-Carvajal et al., 2009]. We adopted the neuroanatomical nomenclature of Marín et al., [1998a] for basal ganglia, Northcutt and Kicliter [1980] for the telencephalon, Neary and Northcutt [1983] for the diencephalon, Wilczynski [1988] for the torus semicircularis 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.

Immunohistochemistry

Túngara frog females (n = 5) were euthanized and their heads rapidly dissected and incubated in 4% paraformaldehyde in 1× PBS (pH 7.4) at 4°C overnight. The heads 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, N.H., USA) in 4 series that were stored at –80°C for 4–6 weeks 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 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 twice 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 (1:500 rabbit anti-TH, or 1:1,000 rabbit anti-DARPP-32) in PBS with 2% normal goat serum and 0.3% Triton-X at room temperature overnight. The polyclonal rabbit anti-TH antibody (Millipore AB152) was raised against rat tyrosine hydroxylase. This is the same antibody used in Yamamoto et al. [2010] and binds to both *th1* and *th2*. The DARPP-32 antibody was purchased from Millipore (AB1656) and was raised against the human DARPP-32 peptide CQVEMIRRRRPTPAM. This human antigen shares 80% identity with the *Xenopus tropicalis* DARPP-32 (Genbank accession number NP_001096342). The portion of the peptide that exactly matches the anuran DARPP-32 is underlined. Sections were then rinsed, incubated for 2 h in a biotinylated goat anti-rabbit secondary antibody (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 substrate (Vector Laboratories). Sections were then Nissl counterstained, dehydrated and cover-slipped with Permount (Fisher Scientific, Itasca, Ill., USA). For control sections, all procedures were the same except that primary antibody was omitted. Due to the method of fixation, cell bodies containing TH or DARPP-32 protein were easily distinguished, but unequivocal identification of TH- or DARPP-32 immunoreactive fibers was not possible.

Western Blot Characterization of Antibodies

In order to determine whether the antibodies would bind specifically to the frog antigen, 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 was 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, incubated in primary antibody (1:2,000 polyclonal rabbit anti-TH, or 1:5,000 DARPP-32 in 1× Tris-buffered saline and 2% NaN_3) for 1 h, washed 5 times for 3 min each in wash buffer, and then incubated in goat-anti-rabbit HRP-conjugated antibody (Southern Biotech) in blocking solution for 30 min. After washing 5 times for 3 min each with wash buffer, the membrane was exposed to HRP substrate (Immobilon Western, Millipore) and exposed to film for 2 min. Using the TH antibody, a single band was visualized at the predicted size of 59–61 kDa, the appropriate size of anuran TH [Chu and Wilczynski, 2002]. Using the DARPP-32 antibody, a single band was visualized at the predicted size of 20.1 kDa, putatively representing DARPP-32 (fig. 1). To predict protein size for DARPP-32, we used *X. tropicalis* DARPP-32 (Genbank accession number NP_001096342) protein sequence and the ExPASy Proteomic server protein molecular weight prediction tool at http://expasy.org/tools/pi_tool.html.

Photomicroscopy

Brightfield optics were used to visualize IHC staining throughout the brain at low (×5) and high magnification (×20). Photographs were taken with a digital camera (AxioCam MRc, Zeiss) attached to an Imager A1 AX10 microscope (Zeiss) using the AxioVision (Zeiss) image acquisition and processing software. Images were compiled and brightness- and contrast-enhanced in Adobe Photoshop CS2.

Results

Here we present the dopamine system in the brain of *P. pustulosus.* Using immunohistochemistry, we characterized the distribution of putative dopaminergic cells containing TH and dopaminoreceptive cells containing DARPP-32. In the following sections we present distribution maps along with representative photomicrographs of

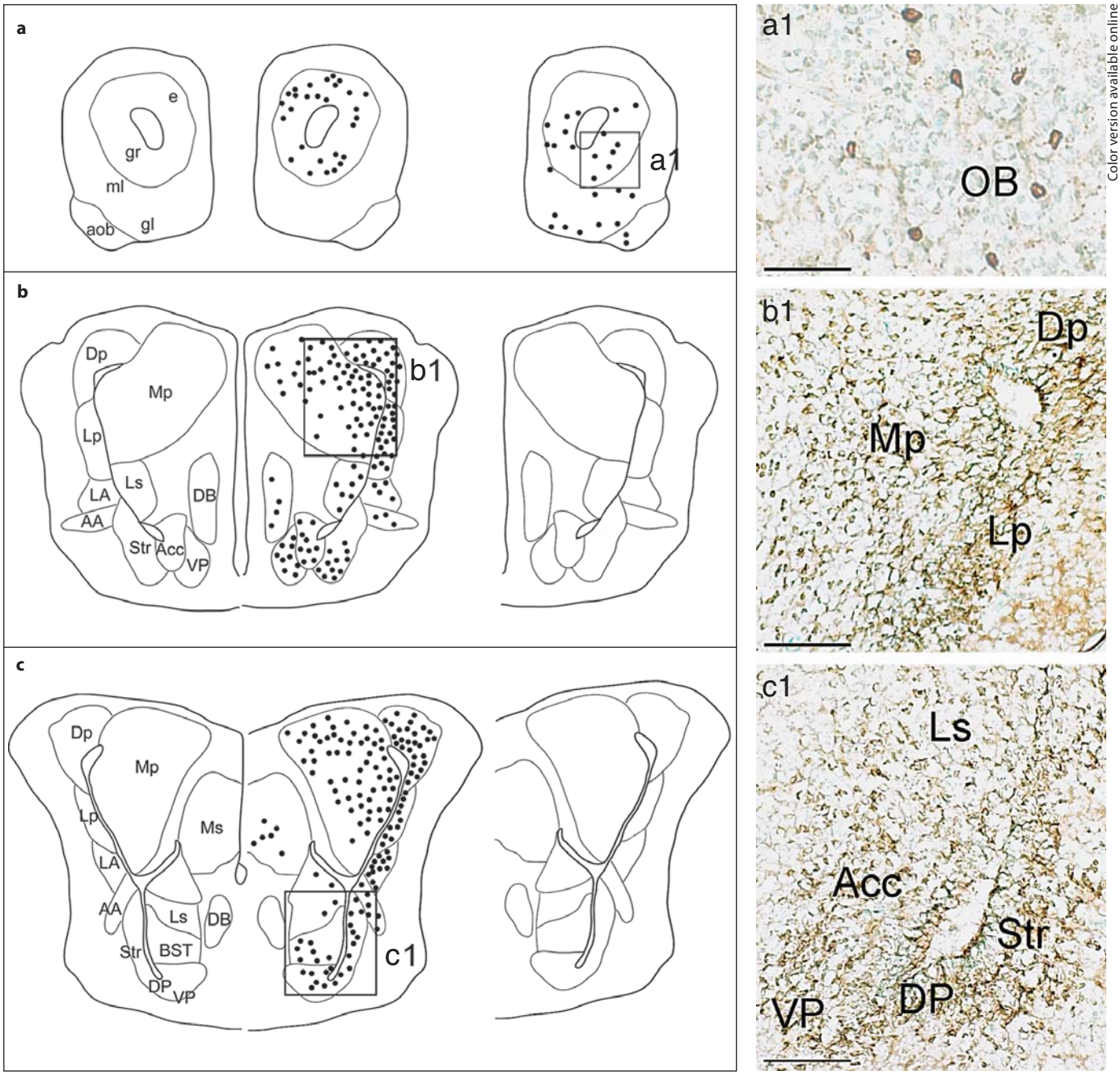


Fig. 2. Distribution of tyrosine hydroxylase and DARPP-32 immunoreactivity in the telencephalon of *P. pustulosus*. Representative sections of the telencephalon are presented as the first image in each panel with DARPP-32 immunoreactive cell bodies shown as dots in the second column and TH-immunoreactive cell bodies shown as dots in the third column. Boxes depict location of micrographs over the sections. The micrograph in the top panel shows TH-immunoreactivity in the olfactory bulb (**a1**). The second panel shows a micrograph of DARPP-32 immunoreactivity in the medial, dorsal, and lateral pallium (Mp, Dp, and Lp, respectively; **b1**). The third panel shows a micrograph of DARPP-32 immunoreactivity in the lateral septum (Ls), nucleus accumbens (Acc), dorsal pallidum (DP), ventral pallidum (VP), and striatum (Str; **c1**). Scale bars = 50 μm.

brain regions for TH and DARPP-32 IHC simultaneously at each level of the brain. For each representative section of the map, the nomenclature is displayed on the left side in the first column, while DARPP-32 protein is represented by dots on the right side in the second column. TH distribution is shown on the right hemisphere in the third column to the right of the DARPP-32 distribution map. Control slides omitting primary antibody showed no staining of cell bodies or fibers for either TH or DARPP-32.

Forebrain

DARPP-32 immunoreactive cells are widely distributed throughout the forebrain while TH immunoreactive cells are restricted to discrete regions in the forebrain (fig. 2). There is an abundance of DARPP-32 protein in the postolfactory eminence (e) and granule layer (gr) of the olfactory bulb, but sparse presence in the mitral and glomerular layers (ml and gl, respectively). There is no DARPP-32 protein in the accessory olfactory bulb (aob). There are a few cells immunoreactive for TH in the granule, mitral, and glomerular layers of the olfactory bulb (fig. 2a1), but the distribution is much more sparse than DARPP-32 immunoreactive cells.

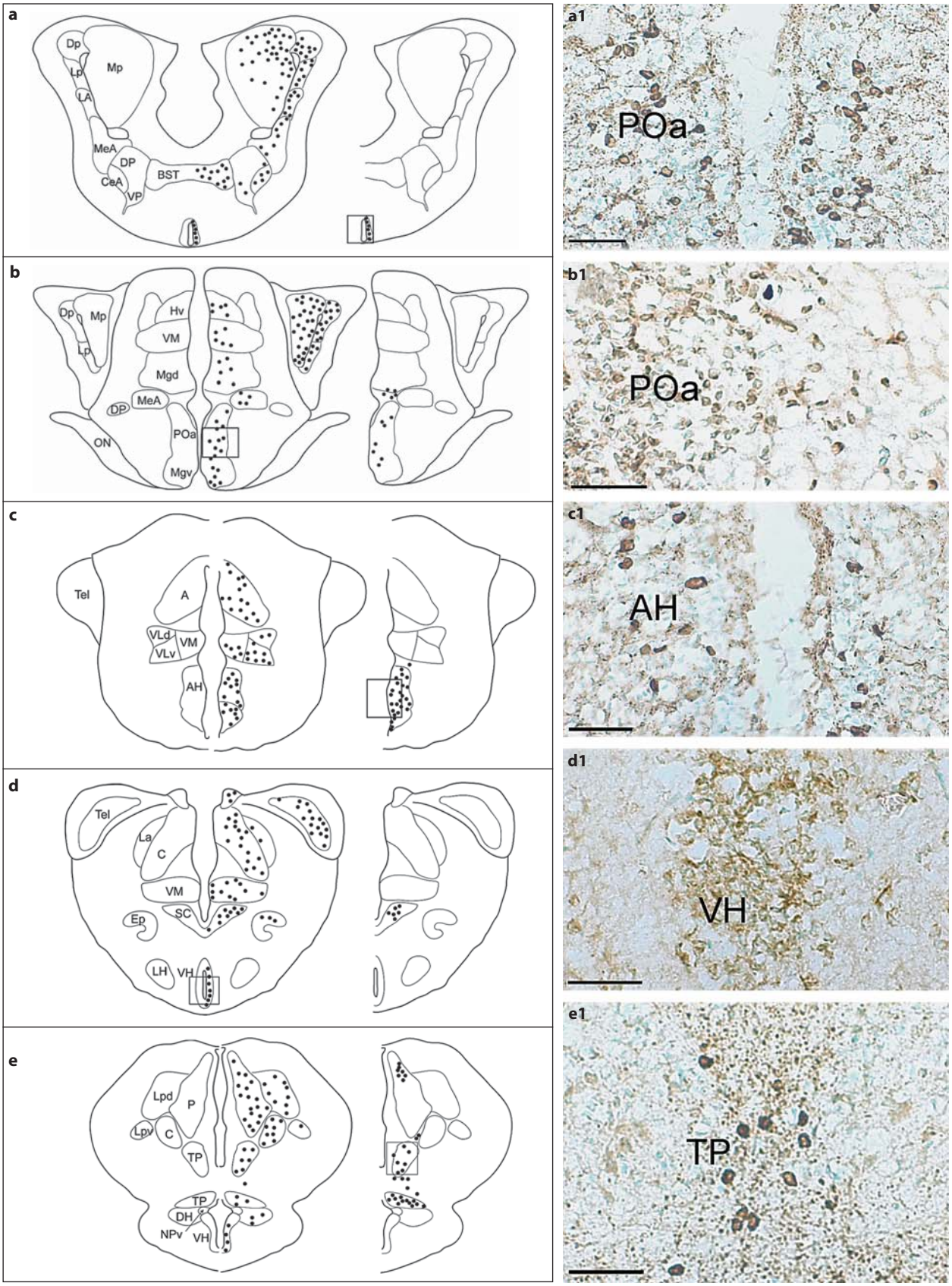
The medial, dorsal, and lateral pallium (Mp, Dp, and Lp, respectively) contain an abundance of DARPP-32 protein (fig. 2b, c). However, these pallial regions do not contain TH immunoreactive cells. The subpallium is situated along the ventral portion of the ventricle. The amygdaloid complex is divided into the lateral amygdala (LA), the anterior amygdaloid (AA), the medial amygdala (MeA), and central amygdala (CeA). All subregions of the amygdaloid complex contain an abundance of DARPP-32 immunoreactive cells, but no TH-immunoreactive cells. Ventral to the amygdaloid complex is the striatum (Str, fig. 2b), which contains an abundance of DARPP-32 cells, but no TH cells. Medial to the striatum is the nucleus accumbens (Acc), which contains an abundance of DARPP-32 immunoreactive cells, but no TH cells (fig. 2c1). The diagonal band of Broca (DB), which runs vertically along the midline, contains DARPP-32 cells but no TH-positive cells. The dorsal and ventral pallidum (DP and VP, respectively) contain DARPP-32 expression, but no TH immunoreactive neurons.

The dorsomedial subpallium is composed of septal nuclei that are divided into lateral and medial regions (Ls and Ms, respectively, fig. 2c). DARPP-32 is present within the lateral septum and more sparsely in the medial septum, although there are no TH-immunoreactive neurons in these regions. Ventral to the caudal septal regions, the rostral portion of the bed nucleus of the stria terminalis (BST) contains DARPP-32 immunoreactive neurons but TH is not present (fig. 2c).

The same general expression patterns continue into the diencephalon where DARPP-32 is widely distributed and TH neurons are restricted to discrete nuclei. The pallial and subpallial regions continue into the diencephalon while the anterior preoptic nucleus (POa) emerges around the third ventricle (fig. 3a) and has the same distribution patterns of DARPP-32 and TH as the more rostral parts in the forebrain. The POa contains both TH (fig. 3a1) and DARPP-32 (fig. 3b1) expression. The POa extends more dorsally and the posterodorsal and posteroventral magnocellular preoptic nucleus (Mgd and Mgv, respectively) emerge, both of which contain an abundance of DARPP-32 immunoreactive cells and a few TH-positive cells (fig. 3b).

Dorsal to the preoptic nuclei, the ventromedial thalamic nucleus (VM) and the ventral habenula (HV) contain DARPP-32 expression but no TH immunoreactivity (fig. 3b). More caudally, the dorsal diencephalon is dominated by thalamic nuclei (fig. 3c). The dorsal and ventral regions of the ventrolateral thalamic nucleus (VLd and VLv, respectively) both contain DARPP-32 immunoreactivity although more abundant in the ventral region. These regions do not contain TH-immunoreactive cells. The anterior thalamic nucleus (A) contains DARPP-32 expression but no TH-positive cells. Ventral to these thalamic regions is the anterior hypothalamus (AH), which contains an abundance of both DARPP-32 immunoreactive cells and TH-positive cells (fig. 3c1). Caudal to the anterior hypothalamus, the ventral hypothalamic nucleus (VH) emerges along the third ventricle and contains

Fig. 3. Distribution of tyrosine hydroxylase and DARPP-32 immunoreactivity in the caudal telencephalon and diencephalon of *P. pustulosus*. Representative sections of the caudal telencephalon and diencephalon are presented as the first image in each panel with DARPP-32 immunoreactive cell bodies shown as dots in the second column and TH-immunoreactive cell bodies shown as dots in the third column. Boxes depict location of micrographs over the sections. The micrograph in the top row shows TH-immunoreactive cell bodies in the anterior preoptic area (POa; **a1**). The micrograph in the second row shows DARPP-32 immunoreactive cell bodies in the POa (**b1**). The micrograph in the third row shows TH-immunoreactive cells in the anterior hypothalamus (AH; **c1**). The micrograph in the fourth row shows DARPP-32 immunoreactive cells in the ventral hypothalamic nucleus (VH; **d1**). The micrograph in the fifth row shows TH-immunoreactive cells in the posterior tuberculum (TP; **e1**). Scale bars = 50 μm.



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DARPP-32 cells (fig. 3d1) but no TH-positive cells. The lateral hypothalamic nucleus (LH) does not contain DARPP-32 or TH-positive cells.

The suprachiasmatic nucleus (SC) emerges dorsal to the third ventricle and contains both DARPP-32 immunoreactive cells and TH expression (fig. 3d). The posterior entopeduncular nucleus (Ep) also contains DARPP-32 immunoreactive cells but no TH-positive cells. The central thalamic nucleus (C) contains DARPP-32 protein, but not TH. The anterior part of the lateral thalamic region (La) contains DARPP-32 immunoreactive cells, but no TH. The posterodorsal and posteroventral regions (Lpd and Lpv, respectively) of the lateral thalamic nucleus both contain DARPP-32 immunoreactive cells, but not TH. The posterior thalamic nucleus (P) is the only thalamic nucleus that contains both DARPP-32 immunoreactive cells and TH protein.

Ventral to the large grouping of thalamic nuclei is the posterior tuberculum (TP), which contains both DARPP-32 immunoreactive cells and an abundance of TH-positive neurons (fig. 3e1). Ventral to the posterior tuberculum and along the third ventricle are the dorsal and ventral hypothalamic nuclei (DH and VH) situated. Both of these hypothalamic regions contain DARPP-32 immunoreactive cells, yet no TH-positive cells. The small nucleus of the periventricular organ (NPv) does not show DARPP-32 or TH protein.

Midbrain and Rostral Hindbrain

DARPP-32 and TH are also present in the midbrain and rostral hindbrain (fig. 4), although the distribution is sparser than the fore- and rostral midbrain. The optic tectum (Tect) contains DARPP-32 protein, but no TH-positive cells (fig. 4a). Ventral to the optic tectum is the torus semicircularis (Tor), which can be divided into 3 distinct clusters [Potter, 1965; Wilczynski, 1988; Hoke et al., 2004]. The laminar nucleus (Tor-L), principal nucleus (Tor-P), and ventral area (Tor-V) all contain DARPP-32 immunoreactive cells but not TH-positive neurons (fig. 4a1).

The anterodorsal and anteroventral tegmental nuclei (Ad and Av, respectively) are ventral to Tor and both contain DARPP-32 protein. There are some TH-positive cells on the dorsal edge of the anteroventral tegmental nuclei. The tegmental nuclei continue more caudally to form the posteriodorsal and posterioventral tegmentum (Pd and Pv, respectively; fig. 4b) neither of which contain TH-positive cells, and only the posterioventral part contains DARPP-32 immunoreactivity. The nucleus reticularis superior (Rs) contains both DARPP-32 and TH protein. More caudally, the cerebellum emerges and contains DARPP-32 protein, but no TH immunoreactivity was observed. The nucleus reticularis medialis (Rm) contains an abundance of DARPP-32 protein (fig. 4c1), but no TH-positive cells. The nucleus motorius nervi trigemini (Vm) and the descending trigeminal tract (Vd) are dorsal to Rm and both contain DARPP-32 protein but not TH immunoreactivity. Finally, the griseum centrale rhombencephali (Gc) has an abundance of DARPP-32 protein, but no TH-immunoreactive cells.

Discussion

In this study, we have used immunohistochemistry to describe the dopaminergic system in the brain of the túngara frog, *P. pustulosus*, a major model system in research on female mate choice and sexual selection [Ryan, 2010]. Specifically, we have mapped out the distribution of dopaminoreceptive cells (with an antiserum raised against DARPP-32) as well as the distribution of putative dopaminergic cells (via immunoreactivity to tyrosine hydroxylase). We have found that there are only a few cell groups that synthesize dopamine as detected by TH immunoreactivity, while cells receiving dopaminergic input are widely distributed throughout the brain as detected by DARPP-32 immunoreactivity. These results not only provide support for the role of dopaminergic modulation of neural circuits in *P. pustulosus* and amphibians in general, but also provide a mechanistic framework to study the dopaminergic modulation of mate choice.

Comparison of the Dopamine System with Other Amphibians

Tyrosine Hydroxylase

Compared to the distribution of DARPP-32 immunoreactive neurons, the distribution of TH-positive cells is much more restricted. We describe here the dopaminergic system in *P. pustulosus* compared with the brains of other anurans, including the African clawed frog (*X. laevis* [González et al., 1993]), the marsh frog (*R. ridibunda* [González and Smeets, 1991]), the fire-bellied toad (*B. orientalis* [Endepols et al., 2006]), and the northern leopard frog (*R. pipiens* [Wilczynski et al., 2003]). Overall the distribution of dopaminergic neurons and fibers is highly conserved across amphibians [González and Smeets, 1994] (table 1). Since DA is a precursor in norepinephrine synthesis, it is possible that TH immunoreactive cells may synthesize norepinephrine rather than dopamine. To confirm that TH-stained bodies are indeed dopami-

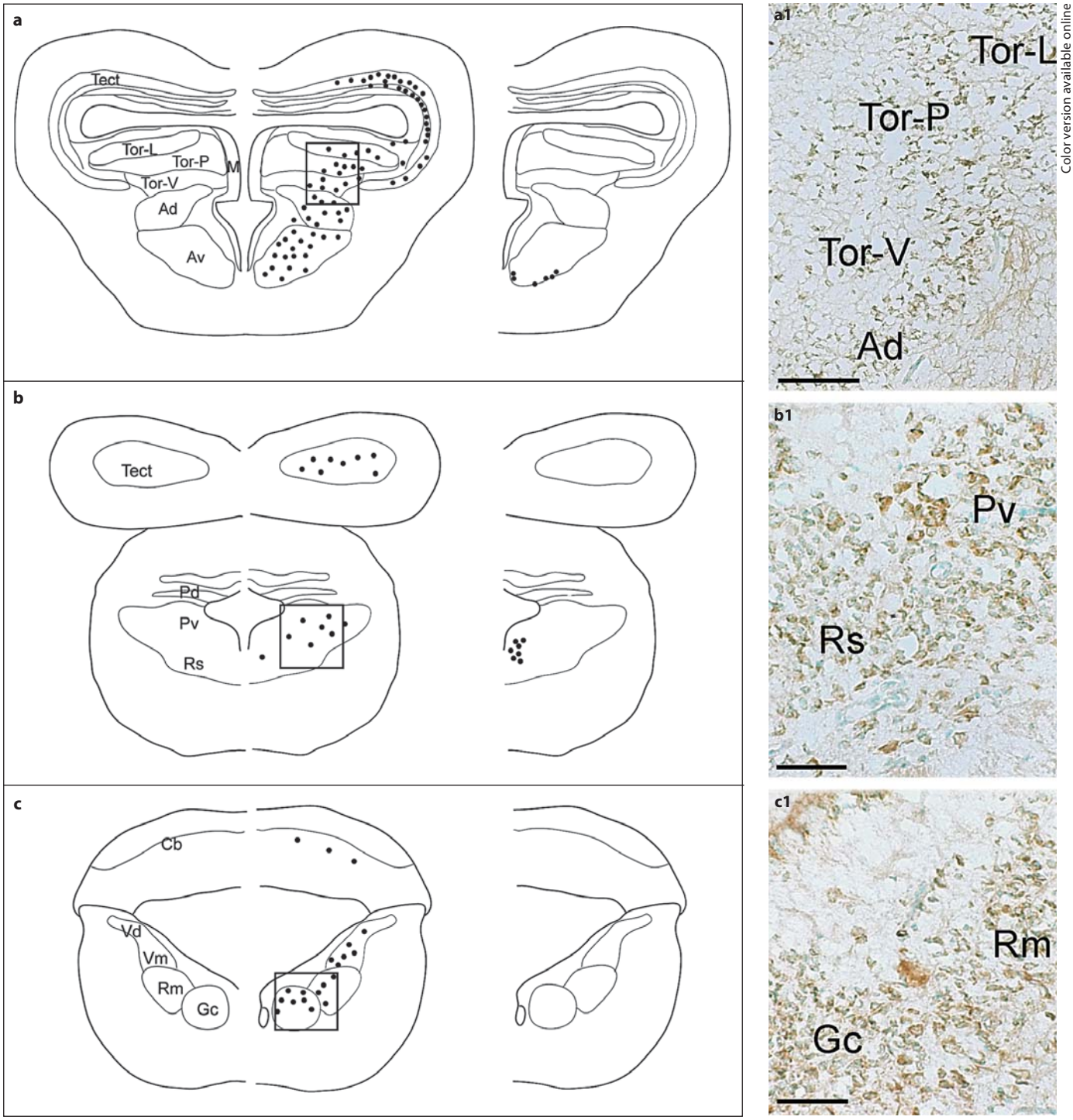

Fig. 4. Distribution of TH-immunoreactivity in the midbrain and rostral hindbrain of *P. pustulosus*. Representative sections of the midbrain and rostral hindbrain are presented as the first image in each panel with DARPP-32 immunoreactive cell bodies shown as dots in the second column of sections and TH-immunoreactive cell bodies shown as dots in the third column of sections. Boxes depict location of micrographs over the sections. The micrograph in the top row shows DARPP-32 immunoreactive cells in the laminar, principal, and ventral area of the toris semicircularis (Tor-L, Tor-P, and Tor-V, respectively) and the anterodorsal tegmental nucleus (Ad; **a1**). The micrograph in the second row shows labeling of DARPP-32 immunoreactive cells in the nucleus reticularis superior (Rs) and nucleus posteroventralis tegmenti (Pv; **b1**). The third panel shows a micrograph of DARPP-32 immunoreactive cell bodies in the nucleus reticularis medius (Rm) and griseum centrale rhombencephali (GC; **c1**). Scale bars = 50 μm.

Table 1. Comparison of TH and DARPP-32 immunoreactivity across amphibians

Brain region	TH cells			TH fibers		DARPP-32
	P. pustulosus	*X. laevis*	*P. waltlii*	*X. laevis*	*P. waltlii*	*P. pustulosus*
Olfactory bulb	+	+	+	+	+	+
Nucleus accumbens (Acc)	–	–	–	+	+	+
Accessory olfactory bulb (aob)	+	+	+	+	+	+
Anteroventral tegmental nucleus (Av)	+	+	+	+	+	+
Bed nucleus of the stria terminalis (BST)	–	–	–	+	+	+
Central thalamic nucleus (C)	–	–	–	+	+	+
Cerebellum (Cb)	–	–	–	+	–	+
Diagonal band of Broca (DB)	–	–	–	+	+	+
Dorsal pallium (Dp)	–	–	–	–	+	+
Griseum centrale rhombencephali (Gc)	–	–	–	+	+	+
Lateral hypothalamic nucleus (LH)	–	–	–	+	+	–
Lateral pallium (Lp)	–	–	–	–	+	+
Lateral thalamic nucleus (Lpv)	–	–	–	+	+	+
Lateral septum (Ls)	–	–	–	+	+	+
Medial amygdala (MeA)	–	–	–	+	+	+
Medial pallium (Mp)	–	–	–	–	+	+
Posterior thalamic nucleus (P)	+	+	+	+	+	+
Anterior preoptic area (POa)	+	+	+	+	+	+
Nucleus posteroventralis tegmenti (Pv)	+	+	+	+	+	+
Suprachiasmatic nucleus (SC)	+	+	+	+	+	+
Striatum (Str)	–	–	–	+	+	+
Toris semicircularis (Tor)	–	–	–	+	+	+
Posterior tuberculum (TP)	+	+	+	+	+	+
Ventral hypothalamic nucleus (VH)	–	–	–	–	+	+
Nucleus motorius nervi trigemini (Vm)	–	–	?	+	?	+
Ventromedial thalamic nucleus (VM)	–	–	–	+	+	+

+ = Cells present; – = no cells; ? = data not available. References: *P. pustulosus*, this study; *X. laevis* and *P. waltlii*, Gonzalez and Smeets [1994].

nergic, we compare our finding to immunohistochemical stains for both TH and DA in *X. laevis* and *R. ridibunda* [González and Smeets, 1990; González et al., 1992].

All amphibians studied so far have TH- and DA-immunoreactive neurons in the olfactory bulb in a distribution pattern similar to *P. pustulosus*. No TH- or DA-positive neurons have previously been reported in the amphibian telencephalon proper, with the exception of the fire-bellied toad *(B. orientalis)*, which is reported to have TH-positive neurons in septal nuclei [Endepols et al., 2006]. The anuran diencephalon contains most of the dopaminergic cells. The dopaminergic cells in the hypothalamus, specifically in the preoptic nuclei and the anterior hypothalamus, are conserved across amphibians. At the rostral chiasmatic level, *P. pustulosus* has TH-positive neurons in the suprachiasmatic nucleus, similar to other amphibians where both TH- and DA-immunoreactivity is observed [González and Smeets, 1994]. Caudal to the suprachiasmatic nucleus, there are TH-positive cell groups in the posterior tuberculum and posterior thalamic nucleus in *P. pustulosus*, which are also seen in other amphibians by both TH- and DA-immunoreactivity. We found some TH-positive cells in the ventral part of the anteroventral tegmental nucleus. This cell group can also be found in other anurans by TH- and DA-immunohistochemistry, but may sometimes be called the nucleus nervi oculomotorii [e.g. González and Smeets, 1994], depending on the nomenclature used. Finally, we found a TH-immunoreactive cell group near the nucleus reticularis superior. This cell group is positive for TH in other anurans as well, but probably consists of noradrenergic neurons, as these cells are noradrenergic, not dopaminergic, in *X. laevis* [González and Smeets, 1994].

Table 2. Comparison of DARPP-32 cell localization across tetrapods

	Frog *(P. pustulosus)*	Pigeon *(C. livia)*	Quail *(C. japonica)*	Gecko *(G. gecko)*	Turtle *(P. scripta)*	Rat *(R. norvegicus)*
Olfactory bulb	+	?	?	+	+	+
Nucleus accumbens	+	+	+	+	+	+
Striatum	+	+	+	+	+	+
Lateral septum	+	+	+	+	–	?
Hippocampus	+	+	+	+	+	+
Amygdala	+	+	+	+	+	+
Preoptic area	+	?	+	+	?	+
Suprachiasmatic nucleus	+	?	+	?	?	?
Thalamus	+	?	+	+	+	+
Dorsal hypothalamus	+	?	+	?	?	?
Lateral hypothalamus	–	?	+	+	?	?
Ventral hypothalamus	+	?	Δ	+	+	+
Torus semicircularis	+	?	?	+	+	?
Ventral tegmental area/substantia nigra	+	?	Δ	Δ	Δ	Δ

+ = Cells present; – = no cells and no fibers; Δ = fibers only; ? = data not available.

References: *frog*, this study; *pigeon*, Durstewitz et al. [1998]; *quail*, Absil et al. [2001]; *gecko*, Smeets et al. [2001]; *turtle*, Smeets et al. [2003]; *rat*, Ouimet et al. [1984], Auger et al. [2001].

DARPP-32

To our knowledge, there is no published description of the distribution of cells receiving dopaminergic input, either by DARPP-32 immunoreactivity or by localization of dopamine D_1 receptors in the amphibian brain. We have found here that dopaminoreceptive cells are widely distributed throughout the brain of *P. pustulosus*. In the following, we discuss the distribution of DARPP-32 immunoreactivity compared to the distribution of DA- or TH-immunoreactive fibers throughout the anuran brain, which appears conserved across species. We also consider our findings in the context of Endepols et al. [2000], who mapped out dopamine D_2 receptors in the auditory midbrain of *X. laevis, D. pictus, and B. orientalis*.

There is an abundance of DA- and TH-immunoreactive fibers in the olfactory bulb in the African clawed frog (*X. laevis* [González et al., 1993]) and the marsh frog (*R. ridibunda* [González and Smeets, 1991]). We have also found DARPP-32 protein here, suggesting that these fibers carry signal to dopaminoreceptive neurons in this region. In the forebrain, there is an abundance of DARPP-32 protein in both the pallium and subpallium of *P. pustulosus*. The majority of TH- and DA-immunoreactive fibers in other anurans in the forebrain are contained in the subpallium, especially in the striatum, lateral septum, and nucleus accumbens. Although we find DARPP-32 protein in the medial, lateral, and dorsal parts of the pallium, there is surprisingly little TH/DA fiber staining in these pallial regions in other anurans. This discrepancy may be due to the use of an antibody in previous studies that only recognized th1-positive fibers, species differences, or sensitivity of the immunohistochemical stains used in those studies.

In the anuran diencephalon and midbrain, TH- and DA- fibers seem to be present in every brain region with the exception of the ventral hypothalamic nucleus (reviewed in [Gonzalez and Smeets, 1994]). We have found DARPP-32 to be in nearly every region in the anuran diencephalon and midbrain, including the ventral hypothalamic nucleus, with the exception of the lateral hypothalamic nucleus. These discrepancies could again be due to antibody specificity to th1, species or sex differences (our study used females while other studies have used males only), as well as technique sensitivity to fiber staining and nomenclatural discrepancies. D_2 receptors in *B. orientalis*, *Discoglossus pictus* and *X. laevis* overlap with the D_1 receptor distribution described here in *P. pustulosus* within many midbrain regions, including the torus semicircularis and tegmentum [Endepols et al., 2000].

In the hindbrain, DARPP-32 was present in most of the brain regions examined with the exception of the nucleus posterodorsalis tegmenti. All of these brain regions contain TH- and DA-immunoreactive fibers in other amphibians [Gonzalez and Smeets, 1994].

Comparison of the Anuran Dopamine System to Other Vertebrates

In the following, we compare the dopamine system of anurans to that of other vertebrates. The distribution of DARPP-32 appears to be highly conserved across vertebrates (table 2), although the possibility that DARPP-32 has taken on a new function in amphibians that has resulted in such widespread distribution, while unlikely, cannot be entirely discounted. As DARPP-32 is widespread in the anuran brain, it is likely involved in a wide range of neural mechanisms, including sensory processing and motor control in addition to regulating social behavior. However, due to the túngara frog's prominent role in research on reproductive decision-making, we focus here on neural networks known to mediate these behavioral processes across vertebrates.

The social behavior network originally described in rodents [Newman, 1999] has now been expanded to other classes of vertebrates including reptiles, birds, and teleosts [Crews, 2003; Goodson, 2005]. Surprisingly, this framework has not been specifically applied to amphibians, although some nodes in this network have been investigated in the context of auditory perception in female túngara frogs [Hoke et al., 2005]. The brain regions in this network are mostly hypothalamic, mediate social behavior, and (by definition) express steroid hormone receptors [Newman, 1999]. Importantly, every node in this network (which includes the preoptic area, anterior hypothalamus, ventromedial hypothalamus, medial amygdala and bed nucleus of the stria terminalis, periaqueductal grey/central grey, and the lateral septum) expresses dopamine D_1 receptors in every major vertebrate lineage studied to date [*mammals:* Weiner et al., 1991; Savasta et al., 1986; Camps et al., 1990; Mansour et al., 1991; Jansson et al., 1999; Hurd et al., 2001; *birds:* Schnabel et al., 1997; Durstewitz et al., 1998; Sun et al., 2000; Absil et al., 2001; *reptiles:* Smeets et al., 2003, 2001; *teleosts:* Kapsimali et al., 2000; O'Connell et al., 2010b]. The only exception seems to be in the avian ventromedial hypothalamus, where presence of dopamine D_1 receptors has not been reported [Kubikova et al., 2010]. We have shown here that the dopaminoreceptive cells are present within each of these brain regions in *P. pustulosus*, suggesting that in amphibians dopamine plays as central a role in modulating social behavior as it does in other vertebrates.

In mammals, the mesolimbic 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 [Deco and Rolls, 2005]. Regions that receive input from this dopaminergic system in mammals include the nucleus accumbens, ventral pallidum, striatum, basolateral amygdala, bed nucleus of the stria terminalis (BNST), hippocampus, and the lateral septum. These brain nuclei contain dopamine D_1 receptors in mammals [Savasta et al., 1986; Camps et al., 1990; Mansour et al., 1991; Weiner et al., 1991; Jansson et al., 1999; Hurd et al., 2001], birds [Schnabel et al., 1997; Durstewitz et al., 1998; Sun et al., 2000; Absil et al., 2001; Kubikova et al., 2010], reptiles [Smeets et al., 2001, 2003], and teleosts [Kapsimali et al., 2000; O'Connell et al., 2010b]. The putative homologies in the amphibian brain to the amniote basal ganglia nuclei are more tentative than those for the pallial telencephalic, hypothalamic or midbrain nuclei and should still be considered with caution until more hodological, neurochemical, developmental, and lesion/stimulation studies are reported. 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 (the lateral amygdala, LA) as a 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; Marin et al., 1995]. 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 the dopaminoreceptive cells are within all of these brain regions in *P. pustulosus*, providing neurochemical support of these suggested homologies to the mammalian mesolimbic reward system.

Given its fundamental role in regulating behavior in mammals, much effort has been put forth to elucidate the anamniote homologue to the mammalian ventral tegmental area. The functional connection between the mammalian ventral tegmental area and nucleus accumbens is considered the foundation of the dopaminergic reward system that associates the salience of an external stimulus with internal cues [Spanagel and Weiss, 1999]. Hodological, neurochemical, and developmental evidence points to the posterior tuberculum as the putative

ventral tegmental area/substantia nigra homologue in anamniotes [Marin et al., 1998b; Rink and Wullimann, 2002]. In both amphibians and teleosts, the posterior tuberculum projects to the putative nucleus accumbens homolog [Marin et al., 1995; Rink and Wullimann, 2001], similar to mammals [Fallon and Moore, 1978]. Although morpholino-knockout studies in zebrafish continue to lend support to this putative homology between the anamniote posterior tuberculum and the mammalian midbrain dopaminergic neurons [Luo et al., 2008], more developmental and neurochemical studies are needed in amphibians. It is unclear, however, whether the posterior tuberculum represents the mammalian ventral tegmental area, substantia nigra, or both, as it is possible that the separation of midbrain dopaminergic cell populations into the anatomically and functionally distinct substantia nigra and ventral tegmental area happened after the anamniotes and amniotes diverged. Once neurochemical markers become available that differentiate the substantia nigra from the ventral tegmental area in mammals, many of these questions can be answered.

Functional Implications of Dopamine in Processing Stimulus Salience

Although no manipulations of dopamine have been done in *P. pustulosus* to allow the assignment of a functional role in female mate choice or male call production, correlations have been drawn between activity in brain regions of the mesolimbic reward system and auditory input using the immediate early gene egr-1. Specifically, female túngara frogs exposed to conspecific male calls exhibited strong egr-1 induction in the posterior tuberculum [Hoke et al., 2005], suggesting that a cellular response in this region may facilitate female-typical behavior in this species. Hoke et al. [2007a,b] also found that egr-1 mRNA levels in the posterior tuberculum were associated with both acoustic stimulation and locomotion and that relative egr-1 levels in basal ganglia regions were significantly correlated with the number of TH-immunoreactive neurons in the posterior tuberculum. Taken together, these results strongly suggest that activity in this region, which represents the putative homolog of the amniote ventral tegmental area/substantia nigra region, may facilitate motivation and/or motor control for phonotaxis in response to an attractive acoustic stimulus.

Lesion studies in other anurans have provided functional insight in to the role of mesolimbic dopamine pathway in female mate choice. Lesions of the striatum abolish phonotactic responses to mating calls in female frogs [Walkowiak et al., 1999]. Additionally, neurotoxic lesions of dopaminergic neurons in the posterior tuberculum disrupts female phonotaxis behavior such that its expression is correlated with the number of TH-neurons remaining in this region [Endepols et al., 2004].

In many vertebrates, dopamine plays an important role in regulating social behavior, including female receptivity and mate-choice, in concert with steroid hormones and neuropeptide pathways. For example, the interaction of dopamine and progesterone has been well studied in mammals, where the progesterone receptor and DARPP-32 are required for both progesterone- and dopamine-facilitated lordosis [Mani et al., 1996, 2000]. We have recently described the distribution of the progesterone receptor in female túngara frogs [O'Connell et al., 2010a]. Interestingly, in this species the distribution of DARPP-32 and the progesterone receptor overlap in many brain regions important for behavioral regulation, suggesting that this pathway may also be important for female mate choice in amphibians. Future work into the endocrine regulation of dopaminergic signaling will provide more insight into to the role of the mesolimbic reward system in regulating social-decision making in anurans.

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Note Added in Proof

Recently, López et al. [2010] described the distribution of DARP-32 immunoreactive cells in the brain of two anurans. Their findings are largely concordant with our results in the túngara frog.

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