

RESEARCH ARTICLE

Dopamine D1Aa and D2a Receptor Expression in the Auditory System of a Vocal Fish

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Received: 12 December 2025 | **Revised:** 31 March 2026 | **Accepted:** 28 April 2026

Keywords: auditory system | D1 receptor | D2 receptor | dopamine receptor | dopamine | fluorescence in situ hybridization | Hybridization Chain Reaction (HCR) | immunohistochemistry | tyrosine hydroxylase

ABSTRACT

Although dopamine receptors (DARs) have been identified in various vertebrate neural circuits, their expression in the central auditory system remains poorly characterized. Reproductive-state changes in catecholamine innervation of central auditory nuclei in the plainfin midshipman fish (*Porichthys notatus*) highlight a unique, ethologically relevant role for catecholamines, including dopamine, in modulating auditory function to enhance reproductive success. Dopamine's effects on these systems are mediated via its receptors; thus, the goal of the present study is to characterize excitatory dopamine *D1Aa* and inhibitory dopamine *D2a* receptor transcript expression throughout the central auditory system of the plainfin midshipman. Fluorescence in situ hybridization-immunohistochemistry (FISH-IHC) revealed robust *D1Aa* and *D2a* expression in forebrain auditory-recipient centers that receive catecholaminergic input: postcommissural and ventral nuclei of the ventral telencephalon, anterior tuberal nucleus, central posterior nucleus of the thalamus, and parvocellular preoptic nuclei. Large dopamine neurons in the periventricular posterior tuberculum, which are, in part, responsible for the reproductive-state changes in central and peripheral catecholamine innervation only express *D2a*, whereas large noradrenergic neurons in the locus coeruleus express both *D1Aa* and *D2a*. The midbrain torus semicircularis, periaqueductal gray, hindbrain octavolateralis efferent nucleus, and descending/secondary octaval nuclei also express both receptor types. *D2a* expression predominates over *D1Aa*, and we identify a subpopulation of cells throughout the auditory system that co-express both receptors. The robust distribution of inhibitory and excitatory DAR expression in the central auditory system, coupled with co-expression in a subset of cells, provides strong neuroanatomical evidence of dopamine's complex role in modulating auditory sensitivity and processing.

1 | Introduction

The neurotransmitter dopamine (DA) acts in the central nervous system of vertebrates to modulate a variety of brain functions, including motivation, reward, reproduction, motor control, learning, and sensory processing (Forlano, Ghahramani, et al. 2015; Hurley et al. 2004; Kehagia et al. 2010; Matsumoto et al. 1999;

Riters 2012). Among these roles, a growing body of evidence supports a modulatory function for dopamine in auditory sensitivity and processing, with its effects mediated via the activation of specific dopamine receptors (DARs) on target neurons (Gittelman et al. 2013; Missale et al. 1998; Toro et al. 2015). DARs are broadly classified into D1-like receptors, which are generally excitatory and stimulate cAMP production, and D2-like receptors, which

are inhibitory and suppress cAMP signaling (Kebabian and Calne 1979; Missale et al. 1998). In addition to their impact on cAMP production, DARs have also been shown to modulate potassium channels and dopamine transporters (DATs), increasing their functional versatility (Liu et al. 1996; Zapata and Shippenberg 2002). Recent studies have implicated a role for central DARs modulating auditory processing, including effects on auditory cortex activity and sensory gain control (Happel et al. 2014; Hoyt et al. 2019) and plasticity (Barr et al. 2021; Macedo-Lima et al. 2021). However, these studies have largely focused on candidate brain regions in mammalian and avian systems, and the broader anatomical distribution of DARs within central auditory circuits, particularly in anamniote vertebrates, remains unclear.

The plainfin midshipman fish (*Porichthys notatus*) is an ideal model for investigating the neural mechanisms underlying auditory function and seasonal plasticity in the auditory system (Bass and McKibben 2003). During the summer months, male midshipman in the intertidal zone of northern CA and the Pacific Northwest excavate nests under rocks and produce an advertisement call to attract females for mating (Brantley and Bass 1994). Females must be able to localize these males by sound to lay their eggs and reproduce (Forlano, Sisneros, et al. 2015). Previous work has shown that females undergo a robust enhancement of hearing sensitivity at the level of the inner ear during the summer months to better encode the higher harmonic frequencies of the hum and thus better detect the mating call (Forlano, Sisneros, et al. 2015; Sisneros 2009). This seasonal enhancement of auditory sensitivity is, in part, caused by both a summer decrease in dopamine innervation and a decrease in dopamine *D2a* receptor transcript expression at the level of the inner ear (Forlano, Ghahramani, et al. 2015; Perelmutter et al. 2019). Although these studies highlight dopamine's modulation of hearing sensitivity at the level of the inner ear, robust seasonal differences in catecholamine (CA) innervation as labeled by fibers immunoreactive for tyrosine hydroxylase (TH; the rate-limiting enzyme in CA synthesis) have also been observed in central auditory nuclei (Forlano, Ghahramani, et al. 2015), presenting an important question of dopamine's (as well as noradrenaline's) function in the central auditory system.

Anatomically, the central auditory system in midshipman is very well characterized (see Bass et al. 2000) (Figure 1) with robust CA innervation throughout its extent (Forlano et al. 2014). Dopaminergic neurons in the periventricular posterior tuberculum (TPp) are responsible for the seasonal change in dopamine innervation of the inner ear, and also send descending projections to the octavolateralis efferent nucleus (OE), descending and secondary octaval nuclei (DO/SO), and ascending projections to the central posterior nucleus of the thalamus (CP), lateral nucleus preglomerulosus (PGl), and some projections to the ventral telencephalon (Vv/Vp/Vs) and parvocellular preoptic area (PPa/PPp) (Forlano et al. 2014; Forlano, Ghahramani, et al. 2015; Perelmutter and Forlano 2017). Furthermore, these TPp neurons indirectly receive auditory input from the auditory midbrain (torus semicircularis) via the periaqueductal gray (PAG) (Kittelberger and Bass 2013), supporting the hypothesis that these neurons and their projections are critically positioned for modulating auditory sensitivity and processing. Although the anatomical distribution of CA innervation is well defined, the

localization of DARs within these central auditory regions has not been previously described in midshipman.

To begin to address dopamine's functional impact on the central auditory system, we compared the expression of an excitatory DAR, *DIAa*, and an inhibitory DAR, *D2a*, in central auditory nuclei in the brain of adult midshipman fish. These DARs were two of seven that were previously identified in the midshipman inner ear (Perelmutter et al. 2019). The sequence of the *DIA* receptor gene is the most conserved in vertebrates, and the *DIAa* subtype in another teleost fish, the zebrafish, is the only *DIA* subtype that shows synteny shared with other species, therefore showing greater conservation of this gene (Yamamoto et al. 2015). Thus, of the excitatory D1-like class of receptors, the *DIAa* receptor gene is an ideal candidate for characterization in the central auditory system. D2 receptors are also highly conserved compared to other D2-like receptors (D3, D4) and exhibit a close phylogenetic relationship with other vertebrate D2 receptors in another teleost fish, the European eel (Pasqualini et al. 2009). Furthermore, because expression of the inhibitory *D2a* receptor is seasonally regulated in the inner ear (Perelmutter et al. 2019), the *D2a* receptor gene is an important candidate for characterization in the central auditory system. Here, we show cells in various central auditory centers that are both innervated by catecholaminergic fibers and express *DIAa* and/or *D2a* receptor transcripts.

2 | Materials and Methods

2.1 | Animals

Female plainfin midshipman fish in reproductive condition ($n = 12$) were collected at low tide by hand in the rocky intertidal zones of Hood Canal, Washington, during the summer breeding season. Reproductive condition was determined on the basis of the presence of females with high gonadosomatic index (GSI) within active nests containing males at the time of collection (Sisneros, Forlano, Knapp, et al. 2004). Standard length (SL) and GSI measurements were recorded for a subset of animals ($n = 10$ of 12; SL mean $\pm$ SD = 16.0 $\pm$ 1.7 cm; range = 13.0–18.5 cm; GSI mean $\pm$ SD = 16.2 $\pm$ 7.2; range = 1.35–25.87). Female GSI varied as some animals had partially spawned at the time of collection. Following collection, animals were shipped to the Aquatic Research and Environmental Assessment Center (AREAC) at City University of New York (CUNY) Brooklyn College. Animals were briefly maintained in recirculating saltwater aquaria between 1 and 5 days before sacrifice. All acquisition, maintenance, and experimental procedures on animals performed in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of CUNY Brooklyn College.

2.2 | Tissue Collection and Preparation

Animals were anesthetized in 0.025% benzocaine solution in seawater and sacrificed via a transcardial perfusion of teleost ringers (4°C) followed by 4% paraformaldehyde (PFA) in phosphate buffer (0.1 M PB, pH = 7.2, 4°C). Brains were dissected, postfixed in PFA for 1 h, rinsed with PB, and cryoprotected in 30% sucrose-PB solution for 48 h at 4°C. Brains were then embedded in

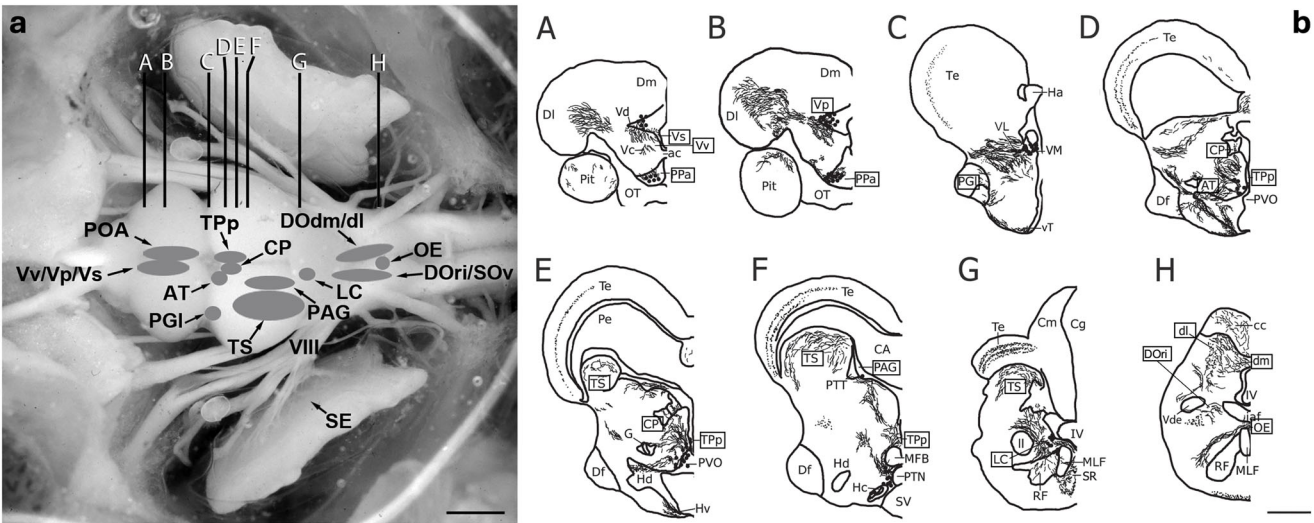


FIGURE 1 | Dorsal view of a midshipman brain (a) with major auditory regions shaded and labeled. Representative series of transverse cross section line drawings (b) illustrating major tyrosine hydroxylase immunoreactive (TH-ir) cell populations, fibers, and terminals. Boxed are auditory and major catecholaminergic regions of interest investigated in the present study. Scale bars = 1.5 mm (a), 500 μm (b). Figures adapted from Forlano, Ghahramani, et al. (2015). ac, anterior commissure; AT, anterior tuberal nucleus; CA, cerebral aqueduct; cc, cerebellar crest; Cg, granular layer of the corpus of the cerebellum; Cm, molecular layer of the corpus of the cerebellum; CP, central posterior nucleus of the thalamus; Df, diffuse nucleus of the hypothalamus; dl, dorsolateral division of the descending octaval nucleus; Dl, lateral zone of area dorsalis of the telencephalon; dm, dorsomedial division of the descending octaval nucleus; Dm, medial zone of area dorsalis of the telencephalon; G, nucleus glomerulosus; Ha, habenula; Hc, central periventricular hypothalamus; Hd, dorsal periventricular hypothalamus; Hv, ventral periventricular hypothalamus; iaf, internal arcuate fiber tract; IV, fourth ventricle; LC, locus coeruleus; ll, lateral lemniscus; MFB, medial forebrain bundle; MLF, medial longitudinal fasciculus; OB, olfactory bulb; OE, octavolateralis efferent nucleus; OT, optic tract; PAG, periaqueductal gray; Pe, periventricular cell layer of the torus semicircularis; PGI, lateral division of nucleus preglomerulosus; Pit, pituitary; POA, preoptic area; PPa, anterior parvocellular preoptic nucleus; PTN, posterior tuberal nucleus; PTT, paratotal tegmentum; PVO, paraventricular organ; RF, reticular formation; SE, saccular epithelium; SOv, secondary octaval nucleus; SR, superior raphe; SV, saccus vasculosus; Te, midbrain tectum; TPp, periventricular posterior tuberculum; TS, torus semicircularis; Vc, central nucleus of area ventralis of the telencephalon; Vd, dorsal nucleus of area ventralis of the telencephalon; Vde, descending tract of the trigeminal nerve; VIII, auditory eighth nerve; VL, ventrolateral nucleus of the thalamus; VM, ventromedial nucleus of the thalamus; Vp, postcommissural nucleus of area ventralis of the telencephalon; Vs, supracommissural nucleus of area ventralis of the telencephalon; vT, ventral tuberal hypothalamus.

TABLE 1 | Hybridization probes and amplification hairpins.

Receptor gene	GenBank accession #	Initiator/Amplifier-fluorescent dye	Lot number
Dopamine D1Aa	GBYQ01030481	B1 Alexa Fluor 488	S020925
Dopamine D2a	GBYQ01052780	B2 Alexa Fluor 594	S017125

Cryo-Gel (Leica Surgipath), sectioned at 20 μm in the transverse plane on a Leica CM1850 cryostat, collected on positively charged microscope slides (Globe Scientific), and stored at −80°C until fluorescence in situ hybridization.

2.3 | Hybridization Probes and Amplification Hairpins

Sequences for the plainfin midshipman dopamine D2a and dopamine D1Aa receptor that were previously identified (Perelmuter et al. 2019) were sent to Molecular Instruments (Los Angeles, California). Hybridization probes with split-initiators (B1 and B2) targeting the specific transcripts were designed and manufactured, and B1 and B2 amplification hairpins were also purchased from Molecular Instruments. Hybridization probes and amplification hairpins used in this study are listed in Table 1.

2.4 | Fluorescence In Situ Hybridization

Slides were removed from −80°C and immediately fixed in 4% PFA (in 0.1 M PB, 4°C) for 15 min, followed by a dehydration series of ethanol (50%/70%/100%/100%) for 5 min each. Slides were briefly soaked in phosphate-buffered saline (0.1 M PBS, pH = 7.2) before a hydrophobic border was applied (Vector Laboratories ImmEdge Pen). Sections were soaked for 2 × 15 min with a solution of 3% hydrogen peroxide (3% H₂O₂, 20 mM NaOH, 0.1 M PBS), washed with PBS 3 × 5 min, and incubated in probe hybridization buffer (Molecular Instruments) for 10 min at 37°C. Probe hybridization solution (16 nM D1Aa and 8 nM D2a in preheated [37°C] probe hybridization buffer) was prepared and applied to slides before an overnight incubation (~20 h) at 37°C. These probe concentrations were optimized in preliminary pilot experiments to achieve an optimal signal-to-noise ratio and were

TABLE 2 | Comparative distribution of dopamine *D1Aa*, dopamine *D2a*, androgen receptor (*AR*), aromatase (*ARO*), estrogen receptor alpha (*ERα*), estrogen receptor beta 1 (*ERβ1*), and estrogen receptor beta 2 (*ERβ2*) in central auditory centers and other areas of interest.

Brain area	D1Aa	D2a	AR	ARO	ERα	ERβ1	ERβ2
Forebrain							
Postcommisural nucleus of the ventral telencephalon (Vp)	++	++	++	+++	+	+	–
Ventral nucleus of the ventral telencephalon (Vv)	++	++	–	+++	+	–	+
Supracommissural nucleus of the ventral telencephalon (Vs)	(–)	+++	+++	+++	+	–	+
Anterior parvocellular preoptic nucleus (PPa)	+	++	+++	+++	+++	+	+
Posterior parvocellular preoptic nucleus (PPp)	++	++	++	+++	–	–	+
Midbrain/Diencephalon							
Periventricular posterior tuberculum (TPp)	–	+++	++	+++	+	–	+
Torus semicircularis (TS)	++	++	++	–	–	–	+
Periaqueductal gray (PAG)	++	+++	+	+	–	+	+
Anterior tuberal nucleus (AT)	+	+	++	+++	+++	–	+
Central posterior nucleus of the thalamus (CP)	+	++	+	+++	+	–	–
Lateral division of the nucleus preglomerulosus (PGL)	(–)	++	+	+	–	–	–
Hindbrain							
Octavolateralis efferent nucleus (OE)	++	++	–	–	–	–	–
Dorsomedial/Dorsolateral division of the descending octaval nucleus (DOdm/dl)	+	++	+	–	–	–	–
Rostral intermediate division of the descending octaval nucleus (DOri)	+	++	–	–	–	–	–
Ventral division of the secondary octaval nucleus (SOv)	+	+++	–	–	–	–	–
Locus coeruleus (LC)	++	+++	–	–	–	–	–

Note: “+++,” “++,” and “+” represent relative levels of hybridization signal of *D1Aa*, *D2a*, *AR*, *Aro*, and *ERα*. Expression of *ERβ1* and *ERβ2* is represented by “+” as relative levels of hybridization signal are unknown. “–” represents zero signal, whereas “(–)” represents signal that is inconclusively above background.

Source: Table modified from Forlano et al. (2010), Fergus and Bass (2013), and Forlano et al. (2016).

used consistently across all tissue sections. After the HCR RNA-FISH hybridization, slides were washed with a series of probe wash buffer (PWB, Molecular Instruments)/saline-sodium citrate Tween buffer (SSCT) at 37°C for 15 min each: 100% PWB/75% PWB/50% PWB/25% PWB. Slides were then washed in 100% SSCT for 15 min at room temperature and treated with amplification buffer (Molecular Instruments) for 1 h. Amplification solution was prepared by heating 6 pmol of hairpin h1 and h2 for both B1 and B2 at 95°C for 90 s, cooling in a dark drawer for 30 min, and then combining all hairpins with amplification buffer. Amplification solution was applied to slides before an overnight incubation (~20 h) at room temperature. Slides were washed 3 × 30 min with SSCT before proceeding to fluorescence immunohistochemistry.

2.5 | Fluorescence Immunohistochemistry

Slides were washed 3 × 10 min in PBS and then blocked for 1 h in PBST (0.1 M PBS, 5% normal donkey serum [NDS, Jackson ImmunoResearch], 0.3% Triton X-100). Primary antibody solution was prepared as in previous studies (Forlano et al. 2014) with mouse anti-TH (1:1000 dilution in PBST, EMD Millipore/Chemicon MAB318, RRID: AB_2201528, Immunogen: TH

purified from PC12 cells, clone LNC1). Primary antibody solution was applied to tissue sections and incubated overnight (~20 h) at room temperature in a humidified chamber. Following this, slides were washed for 5 × 10 min in 0.1 M PBS + 0.5% NDS and incubated with Alexa Fluor 680 diluted in PBST (1:200, anti-mouse Alexa Fluor 680, Invitrogen) for 2 h. Finally, slides were washed 4 × 10 min in PBS and cover slipped with Prolong Gold Antifade Reagent with 4,6-diamidino-2-phenylindole (DAPI) nuclear counterstain (Invitrogen).

2.6 | Fluorescent Image Acquisition

All images were taken on an Olympus BX61 epifluorescence compound microscope using various objectives (20×, 40×, 60×) with DAPI, GFP, Texas Red, and Cy5.5 (ultra far red) filters (Chroma). Images were acquired with Metamorph imaging software (Molecular Devices) as 5–11 μm z-stacks with 0.2–1 μm steps depending on the objective. Exposure time, number of z-stacks, and step size were all optimized for each image and region of interest. Photomicrographs were compiled via a maximum intensity projection of each channel, contrast-enhanced on Metamorph or ImageJ, and overlaid into a single image.

3 | Results

3.1 | Fluorescence In Situ Hybridization-Immunohistochemistry (FISH-IHC) Overview

An anatomical overview of all auditory regions sampled is indicated in Figure 1. A summary of *DIAa* and *D2a* expression with relative abundance in central auditory regions is provided in Table 2, which also highlights the expression of androgen and estrogen receptors, as well as aromatase, which converts testosterone to estrogen (Fergus and Bass 2013; Forlano et al. 2001, 2005, 2010, 2016).

3.2 | Telencephalon

Robust labeling of *DIAa* and *D2a* occurs throughout the area ventralis (V) and preoptic area (PPa/PPp). Although these regions are not exclusively auditory in function, they receive input projections from auditory nuclei and the TPp (Bass et al. 2000; Forlano et al. 2014; Goodson and Bass 2002) and are therefore included in this study. Within the postcommissural (Vp) and ventral nuclei (Vv), many cells co-express *DIAa* and *D2a* (Figure 2a–f). DAR expression is largely uniform throughout the entirety of both nuclei. *D2a* seems to predominate over *DIAa* within cells that co-express *DIAa/D2a*, and there is a population of cells in both nuclei that express *D2a* but not *DIAa* (Figure 2a–f). Within the supracommissural nucleus (Vs), cells express *D2a* (Figure 2g); however, *DIAa* signal is not above background (Figure S1). All of these regions are potential targets of the local TH-ir cell population (shown in Figure 2a), which also express *D2a*, and the TPp (Forlano et al. 2014; Northcutt 2006; Tay et al. 2011). Furthermore, within the anterior and posterior parvocellular preoptic nuclei (PPa/PPp), many cells co-express *DIAa* and *D2a* (Figure 3a–f) with a uniform robust expression throughout the nuclei.

3.3 | Periventricular Posterior Tuberculum

There is robust *D2a* expression in the large TH-ir cells of the periventricular posterior tuberculum (TPp, Figure 4a,b), which provides dopaminergic efferents to the peripheral auditory system as well as all levels of the central auditory system (Forlano et al. 2014; Perelmuter and Forlano 2017). Within the entire brain, *D2a* expression is perhaps the most robust in these dopaminergic TPp cells and is one population that we identified to conclusively not express *DIAa*. Although the TPp broadly has high *D2a* expression relative to other regions, there is some variation of expression within each individual TH-ir TPp cell. Despite this, the expression pattern and variation seem uniform throughout the rostral-caudal length of the nucleus.

3.4 | Auditory Diencephalon and Midbrain

Robust labeling of *DIAa* and *D2a* occurs throughout the auditory midbrain/diencephalon. Within the torus semicircularis nucleus centralis (TSnc), the main central auditory relay center to higher order nuclei (Bass et al. 2000), and the periaqueductal gray (PAG),

an auditory-vocal integration site with reciprocal connections with the TPp (Kittelberger and Bass 2013), there are a subset of cells that co-express *DIAa* and *D2a* and another subset of cells that only express *D2a* (Figure 5a–g). Within the anterior tuberal nucleus (AT) and central posterior nucleus of the thalamus (CP), auditory regions that receive input from both the TS and TPp (Bass et al. 2000; Forlano et al. 2014), there also seems to be a subset of cells that co-express *DIAa* and *D2a*, and another subset of cells that only express *D2a* (Figure 6a–j). Furthermore, TH-ir cells in the AT seem to show a very robust expression of *D2a* (Figure 6a). In the nucleus preglomerulosus (PGl), which also receives input from the TS, there is a subset of cells that express *D2a* (Figure 6k). However, as seen in Vs, *DIAa* signal is not conclusively above background. Interestingly, in all these regions, there seems to be great variation of receptor expression within cells (see arrowheads in Figure 6k). Despite this, the expression pattern and variation seem uniform throughout the rostral-caudal length of these nuclei.

3.5 | Hindbrain

In the octavolateralis efferent nucleus (OE), which sends cholinergic projections to the inner ear (Bass et al. 1994; Highstein and Baker 1986) and receives its sole catecholaminergic input from dopamine originating from the TPp (Perelmuter and Forlano 2017), a subset of cells appear to co-express *DIAa* and *D2a* (Figure 7a,b). This is consistent in both the caudal (OE_c) and rostral (OE_r) subdivisions. In the locus coeruleus (LC), which is the primary source of noradrenaline and receives dopaminergic input from the TPp (Kaslin and Panula 2001), all cells seem to co-express *DIAa* and *D2a* (Figure 7c–e), which is consistent throughout the rostral-caudal length of the LC.

At all three levels of the descending octaval nucleus (dorsomedial–DO_{dm}, Figure 8a–c; dorsolateral–DO_{dl}, Figure 8d–f; and rostral intermediate–DO_{ri}, Figure 8g–i), as well as the secondary octaval nucleus (SO_v, Figure 8j–l), there is a subset of cells with robust co-expression of *D2a* and *DIAa*. Both the DO and the SO receive input from primary auditory afferents and are densely innervated by TH-ir fibers, likely originating from the TPp (Bass et al. 2001; Forlano et al. 2014). All these regions also contained a subset of cells that only expressed *D2a*, and we did not identify any cells that solely expressed *DIAa*. Within the three levels of the DO, the expression pattern was consistent throughout the nuclei proper. Within the SO_v, expression of both receptors seemed to increase with the higher TH-ir innervation in the rostral part of the nucleus.

4 | Discussion

4.1 | Overview

In this study, we characterized dopamine *DIAa* and *D2a* receptor transcript mRNA distribution throughout the central auditory system of the plainfin midshipman fish. To our knowledge, this is the first study to broadly characterize both excitatory and inhibitory DAR expression together in an auditory system of a vertebrate as well as demonstrate robust co-expression of excitatory and inhibitory DARs within individual neurons

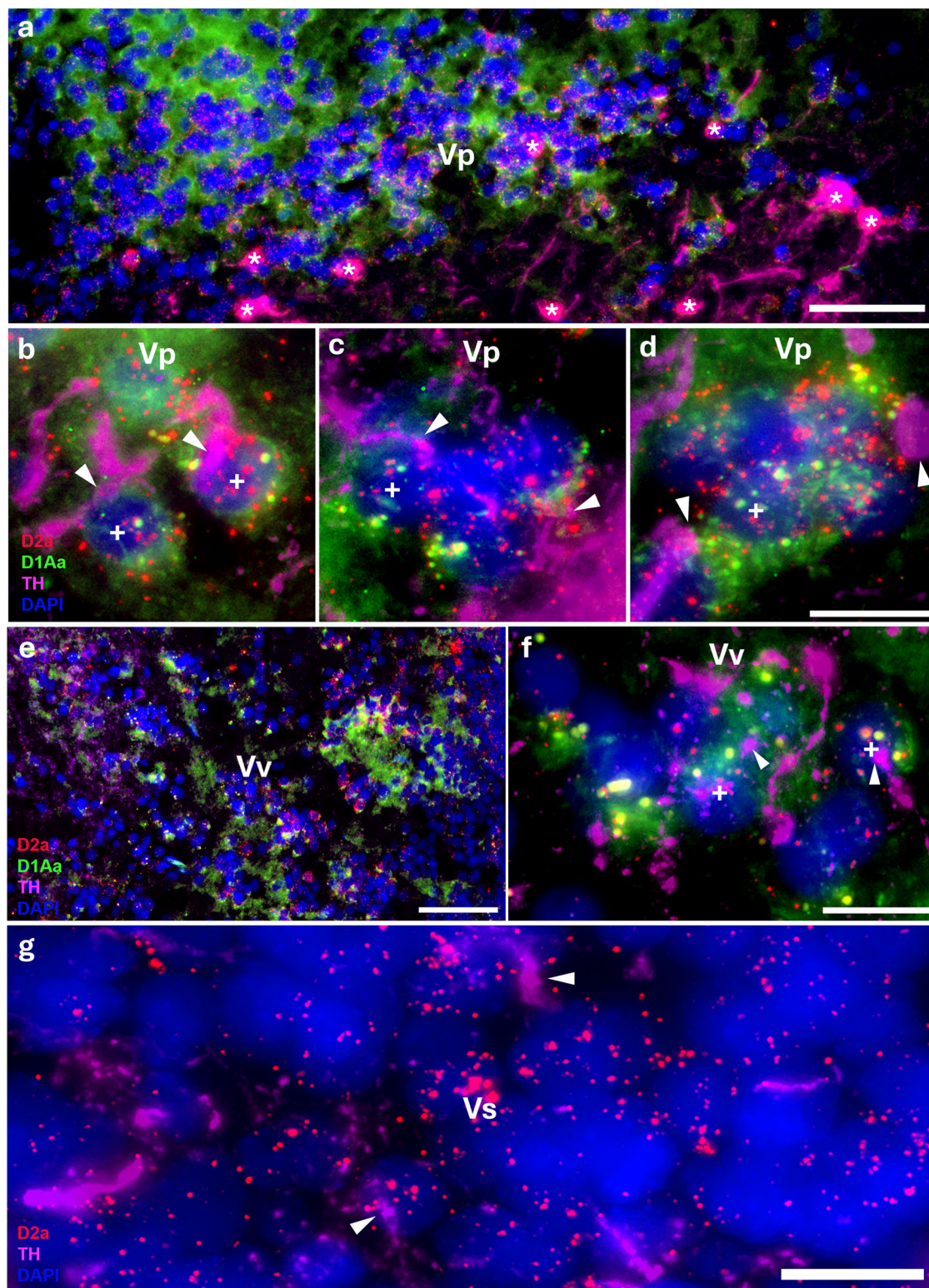


FIGURE 2 | Robust *D1Aa* (green)/*D2a* (red) expression and TH-ir (magenta) innervation of forebrain nuclei; nuclei stained with DAPI in blue. (a–f) TH-ir fibers innervating/terminating at postcommissural nucleus of area ventralis (Vp) cells (a–d) and ventral nucleus of area ventralis (Vv) cells (e and f) co-expressing *D1Aa* and *D2a* receptor transcripts (labeled with +). TH-ir cell population lies just ventral to the postcommissural nucleus of area ventralis (Vp, a, * denotes TH-ir cell). (g) TH-ir fibers innervating/terminating at supracommissural nucleus of area ventralis (Vs) cells expressing *D2a* receptor transcripts. All arrowheads point to TH-ir fibers/putative terminals. Scale bars in a, e = 50 μ m; b, c, d, f, g = 10 μ m. DAPI, 4,6-diamidino-2-phenylindole; TH, tyrosine hydroxylase.

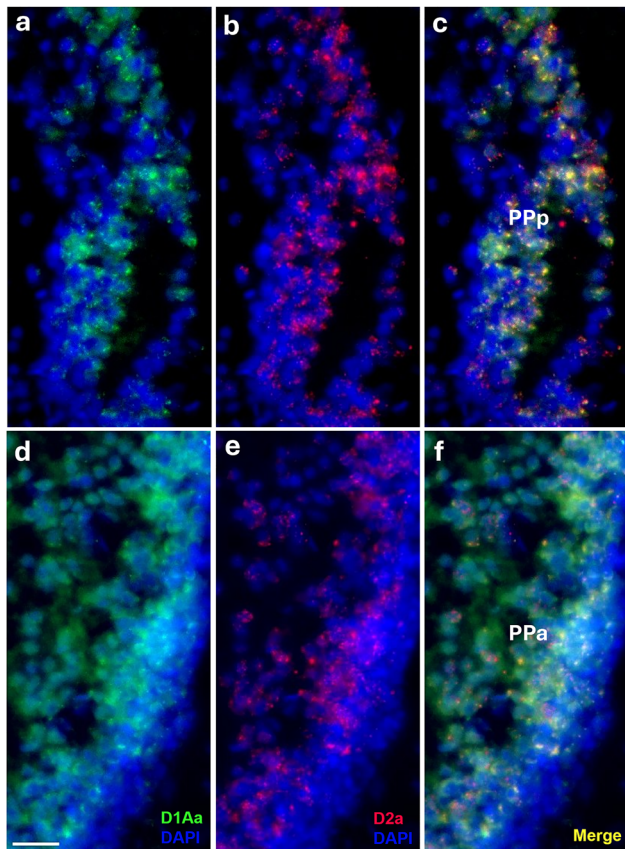


FIGURE 3 | Robust *D1Aa* (green)/*D2a* (red) expression in the posterior parvocellular preoptic area (PPp, a–c) and the anterior parvocellular preoptic area (PPa, d–f); nuclei stained with DAPI in blue. Scale bar = 20 μ m. DAPI, 4,6-diamidino-2-phenylindole.

spanning hindbrain to forebrain. Furthermore, by combining FISH with traditional IHC to label for TH, we provide strong neuroanatomical support for dopamine innervation and modulation in specific auditory nuclei via its receptor expression. Although TH is a marker for both dopaminergic and noradrenergic cells and fibers, our previous studies have demonstrated that TPp neurons, known to be dopaminergic (see below), project specifically to several auditory nuclei, and the auditory hindbrain is largely devoid of noradrenergic fibers (Forlano et al. 2014; Perelmuter and Forlano 2017). Although we focused primarily on two DARs in the central auditory system (see Section 1), future studies characterizing the central expression of the other five DARs in midshipman are needed to better understand how dopamine modulates auditory sensitivity and processing, as well as its role in neuroendocrine, motivational, and motor functions. Furthermore, a quantitative/seasonal analysis of DAR expression was beyond the scope of this initial study and will be reported elsewhere.

4.2 | *D2a* Expression in the Periventricular Posterior Tuberculum

The TH-ir neurons in the periventricular posterior tuberculum (TPp) show the most robust expression of *D2a* throughout the entire brain. Many studies confirm that these cells are indeed DAergic as they are immunoreactive to DA and TH but not

dopamine β -hydroxylase and contain genetic markers specific for dopamine neurons in mammals (Batten et al. 1993; Ekström et al. 1986; Filippi et al. 2010; Forlano et al. 2014; Kaslin and Panula 2001; Perelmuter and Forlano 2017; Ryu et al. 2007; Yamamoto et al. 2011). Furthermore, previous work has shown that these TPp neurons send strong descending projections to the hindbrain, local projections within the diencephalon, and ascending projections to the ventral telencephalon—all regions that were investigated in this study (Forlano et al. 2014, 2017; Ma 2003). Seasonal changes in CA innervation of various central auditory centers as well as the inner ear are either entirely or in part caused by the TPp (Forlano, Ghahramani, et al. 2015; Perelmuter et al. 2021), thus strongly implicating an important role for these dopaminergic cells in modulating auditory functions.

Interestingly, we did not observe any *D1Aa* expression in the dopaminergic neurons of the TPp. Although a previous study found *D1A* and *D2* mRNA in the TPp of African cichlids (O'Connell et al. 2011), it was not confirmed if *D1A* was localized to the dopaminergic cells within the TPp. We labeled for the more specific *D1Aa* subtype in the present study, and ultimately the midshipman *D1Ab* and *D1Ba* subtypes must be tested to confirm the lack of D1 receptors in the dopaminergic cells of the TPp. Lastly, we believe that, by labeling TH-ir TPp neurons coupled with both receptors, we have higher spatial resolution that confirms DAR mRNAs localized to individual cells.

There is currently little to no neuroanatomical evidence for dopamine cells in the TPp receiving dopaminergic input from another brain region. Thus, the robust presence of *D2a* receptor transcript mRNAs on TPp cells suggests a strong autoregulatory role coupled with presynaptic regulation. In rodents, somatodendritic dopamine release in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) has been shown to activate D2 autoreceptors on dopamine neurons, which leads to inhibition via activation of G-protein-coupled inwardly rectifying potassium (GIRK) channels (Aghajanian and Bunney 1977; Beckstead et al. 2004; Courtney et al. 2012; Lacey et al. 1987; Mercuri et al. 1997). Furthermore, D2 autoreceptors can increase the activity of dopamine transporter (DAT), thus decreasing dopamine available for postsynaptic signaling (Cass and Gerhardt 1994; Dickinson et al. 1999; Wu et al. 2002). D2 autoreceptors can also inhibit TH, which is proposed to be a slower, long-lasting mechanism via an inhibition of adenylyl cyclase (AC), which leads to a reduction of cAMP binding to protein kinase A in the regulatory domain of TH (Ford 2014; Harada et al. 1996; Lindgren et al. 2001; Onali and Olanas 1989). D2Rs can also serve as presynaptic autoreceptors on axon terminals to inhibit the likelihood of vesicular dopamine release (Benoit-Marand et al. 2001; Suaud-Chagny et al. 1991). Altogether, if these functions are largely conserved, the high expression of *D2a* in these widely projecting neurons provides support for the regulation of dopaminergic signaling at multiple levels.

Another finding from the present study is the variation in relative expression of *D2a* within individual TH-ir cells in the TPp (see Section 3.3, Figure 4b). Although we did not run a quantitative analysis, the variation in *D2a* expression may reflect natural heterogeneity in receptor expression; however, it is also possible that there is differential *D2a* expression in various dopaminergic

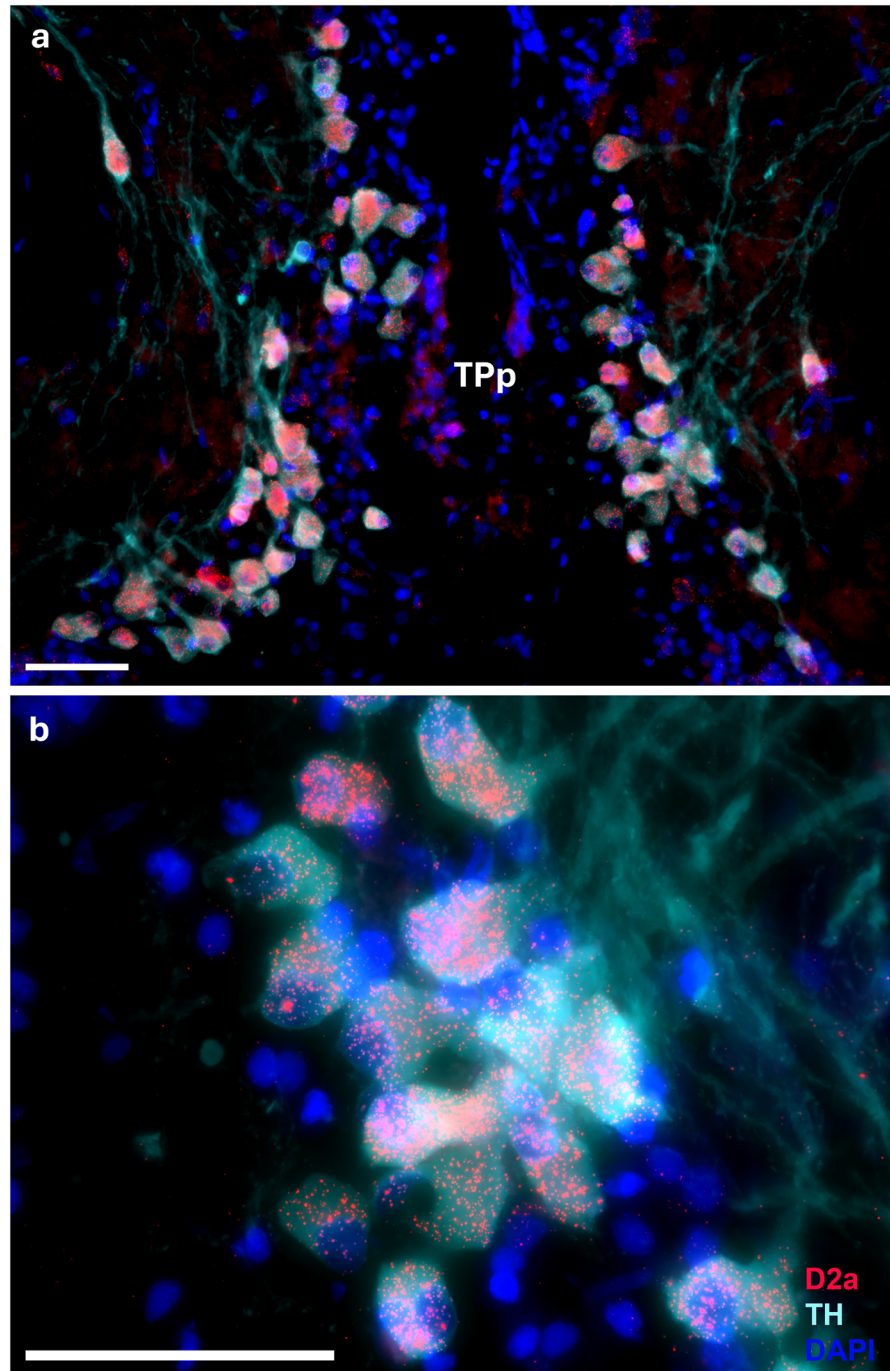


FIGURE 4 | Robust *D2a* expression (red) on somata of large dopamine neurons labeled by TH-ir (cyan) in the periventricular posterior tuberculum (TPp); nuclei stained with DAPI in blue (a: low-magnification image, 20× objective; b: high-magnification image, 60× objective, from the right-hand side of the region shown in Panel a). Scale bars = 50 μm in both panels. DAPI, 4,6-diamidino-2-phenylindole; TH, tyrosine hydroxylase.

TPp cells that have different downstream targets. For instance, a catecholamine projectome study in larval zebrafish has demonstrated that a certain subset of TPp neurons project to various targets—a small percentage ascend to the ventral telencephalon (e.g., Vv/Vd), whereas far more project to the hypothalamus and hindbrain/spinal cord (Tay et al. 2011). In midshipman fish, approximately 5%–10% of TH-ir TPp neurons project to the inner ear (Forlano et al. 2014). Furthermore, testosterone treatment only affected a subset of TH-ir projections that originate from

the TPp, indicating that only some TPp neurons are sensitive to androgens (Perelmuter et al. 2021). Although it is unknown if individual dopamine cells in the TPp have extremely widespread projections (e.g., both ascending and descending) in midshipman, the low percentages of TPp neurons that project to specific regions perhaps suggest a more specialized role of each individual TPp neuron. Ultimately, a quantitative tract-tracing study coupled with labeling *D2a* and TH is needed to determine if TPp cells that project to specific targets differentially express the *D2a* receptor.

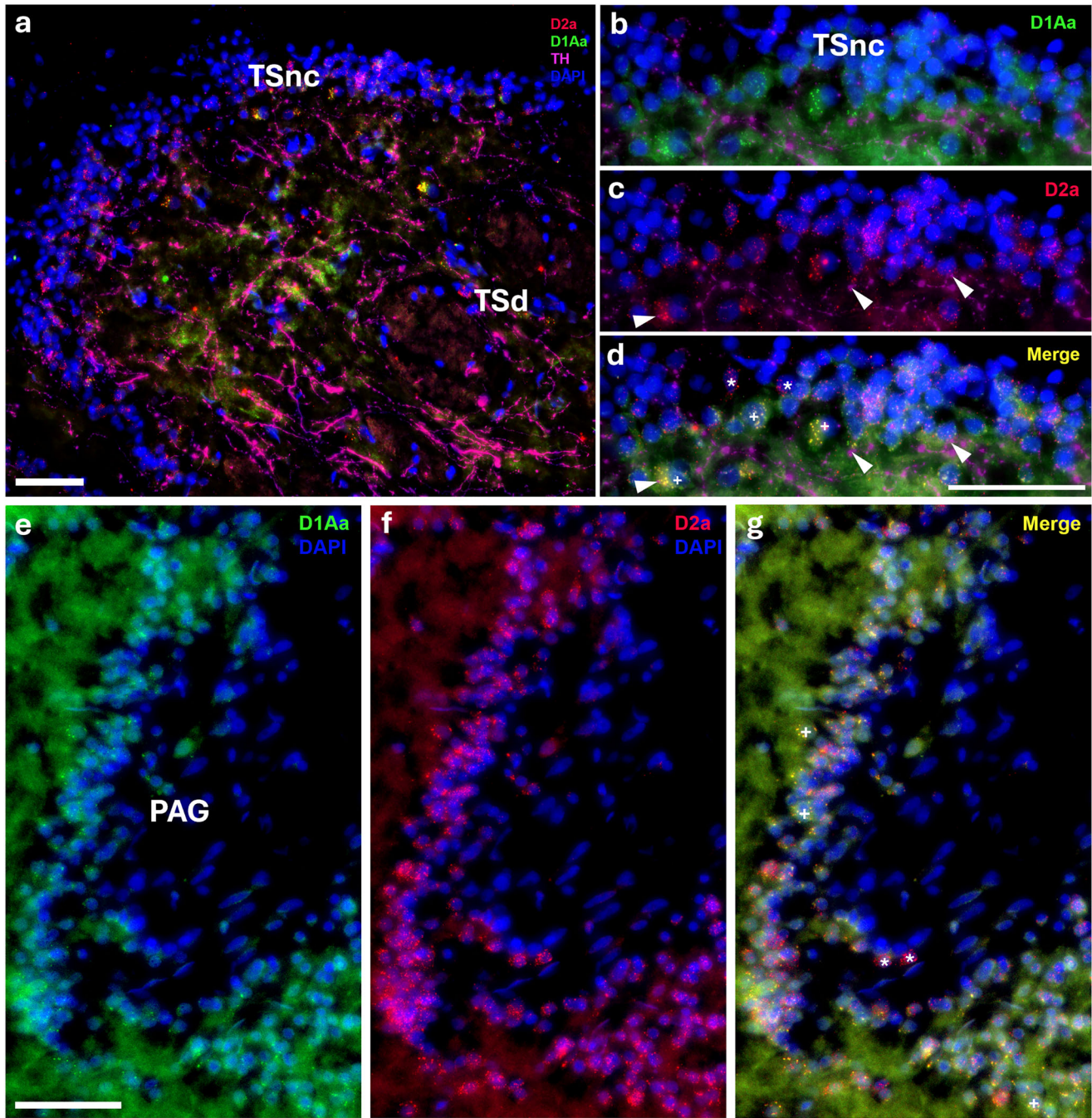


FIGURE 5 | Robust *D1Aa* (green)/*D2a* (red) expression and TH-ir (magenta) innervation of midbrain nuclei stained with DAPI in blue. (a–d) TH-ir fibers innervating/terminating at torus semicircularis nucleus centralis (TSnc) cells co-expressing *D1Aa* and *D2a*. (e–g) Robust *D1Aa*/*D2a* expression in periaqueductal gray (PAG) cells. Arrowheads in (c) and (d) point to TH-ir fibers/putative terminals. + labeled cells are *D1Aa*+/*D2a*+ and * labeled cells are *D1Aa*–/*D2a*+. Scale bars in a, e, f, g = 50 μm; b, c, d = 10 μm. DAPI, 4,6-diamidino-2-phenylindole; TH, tyrosine hydroxylase.

4.3 | *D1Aa* and *D2a* Expression in the Telencephalon

The area ventralis (V) contains nodes that are both auditory recipient as well as part of the vertebrate social behavior network (Goodson 2005; O'Connell and Hofmann 2011, 2012). In the present study, we found robust expression of *D1Aa* and *D2a* receptors in the Vp and Vv, and only *D2a* in the Vs. Vp and Vs are proposed to be homologous to the mammalian extended central

amygdala/bed nucleus of stria terminalis and the Vv is proposed to be homologous to the septum (Bruce and Braford 2009; Goodson and Kingsbury 2013; Maximino et al. 2013; Northcutt 2008). These regions receive a minority of their input from the Tpp and likely undergo complex regulation via dopamine from multiple sources for functions that may not be primarily auditory-related (Forlano et al. 2014). Similarly, we observed robust co-expression in both the anterior and posterior parvocellular divisions of the POA, another critical node of the social behavioral

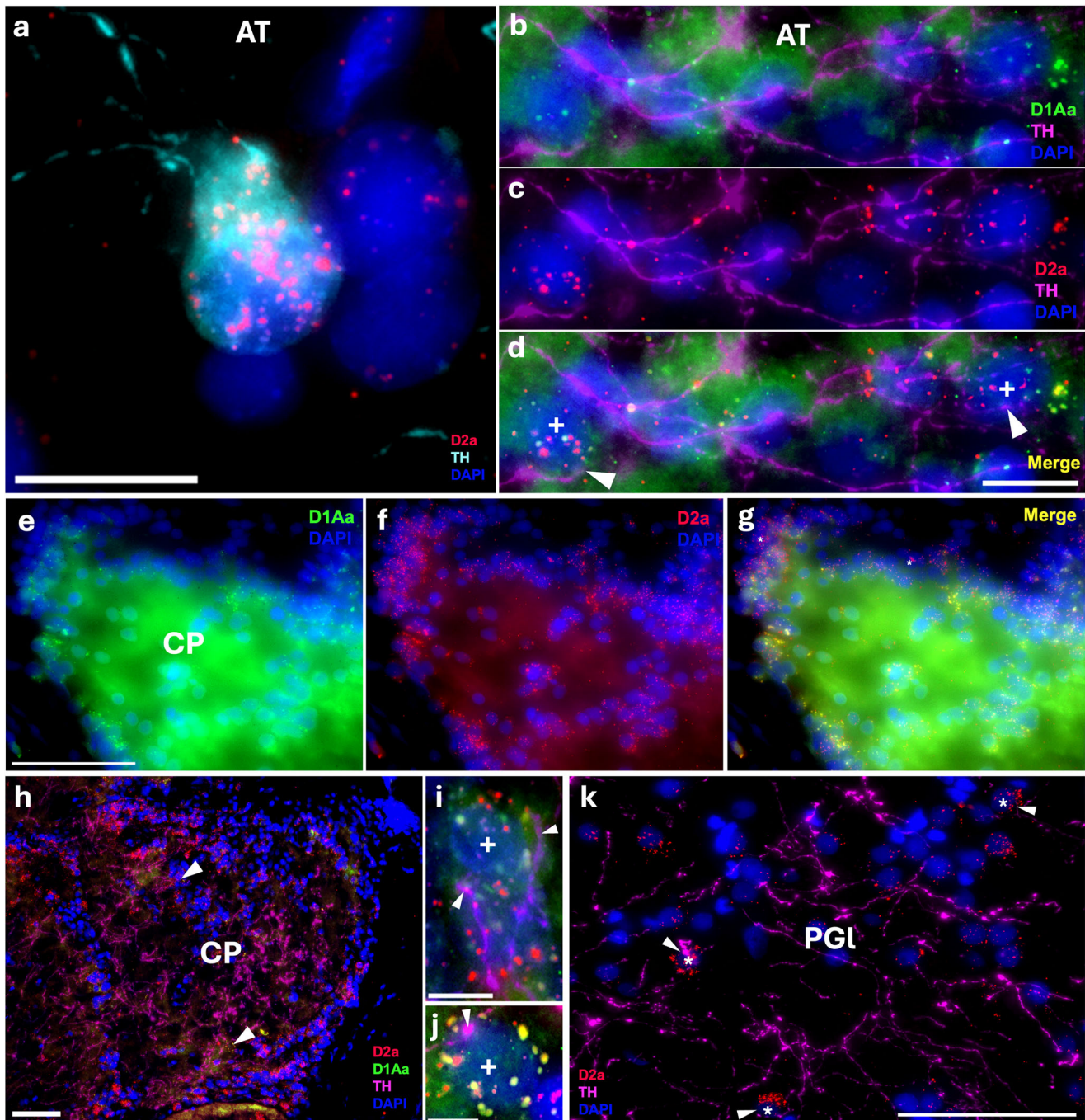


FIGURE 6 | Robust *D1Aa* (green)/*D2a* (red) expression and TH-ir (magenta) innervation of diencephalic/midbrain auditory centers; nuclei stained with DAPI in blue. (a) TH-ir cell (cyan) in the anterior tuberal nucleus (AT) expressing *D2a* (red). (b–d) Other cells in the AT surrounded by TH-ir fibers (magenta) and co-expressing *D1Aa* and *D2a*. (e–g) Cells in the central posterior nucleus of the thalamus (CP) co-expressing *D1Aa* and *D2a*. (h–j) TH-ir fibers innervating/terminating at CP cells co-expressing *D1Aa* and *D2a*. (k) TH-ir fibers innervating/terminating at lateral nucleus preglomerulosus (PGL) cells expressing *D2a*. + labeled cells are *D1Aa*+/ *D2a*+/ and * labeled cells are *D1Aa*–/ *D2a*+. All arrowheads point to TH-ir fibers/putative terminals; arrowheads in (h) point to TH-ir fibers/putative terminals on cells that are shown in (i) and (j). Scale bars in a, b, c, d = 10 μ m; e, f, g, h, k = 50 μ m; i, j = 5 μ m. DAPI, 4,6-diamidino-2-phenylindole; TH, tyrosine hydroxylase.

network (Goodson 2005; O’Connell and Hofmann 2011). Both of these findings support a previous study done in a cichlid fish reporting both *D2* and *D1a* receptor expression in the Vv and POA (O’Connell et al. 2011). Dopaminergic modulation within the POA has been shown to regulate social and reproductive behaviors across vertebrates (Dufour et al. 2005; Hull et al. 1995; Kleitz-Nelson et al. 2010; Nutsch et al. 2016), suggesting that receptor expression in the POA likely reflects a broader integrative role beyond auditory processing. As such, these functions are outside the scope of this article and will be described elsewhere.

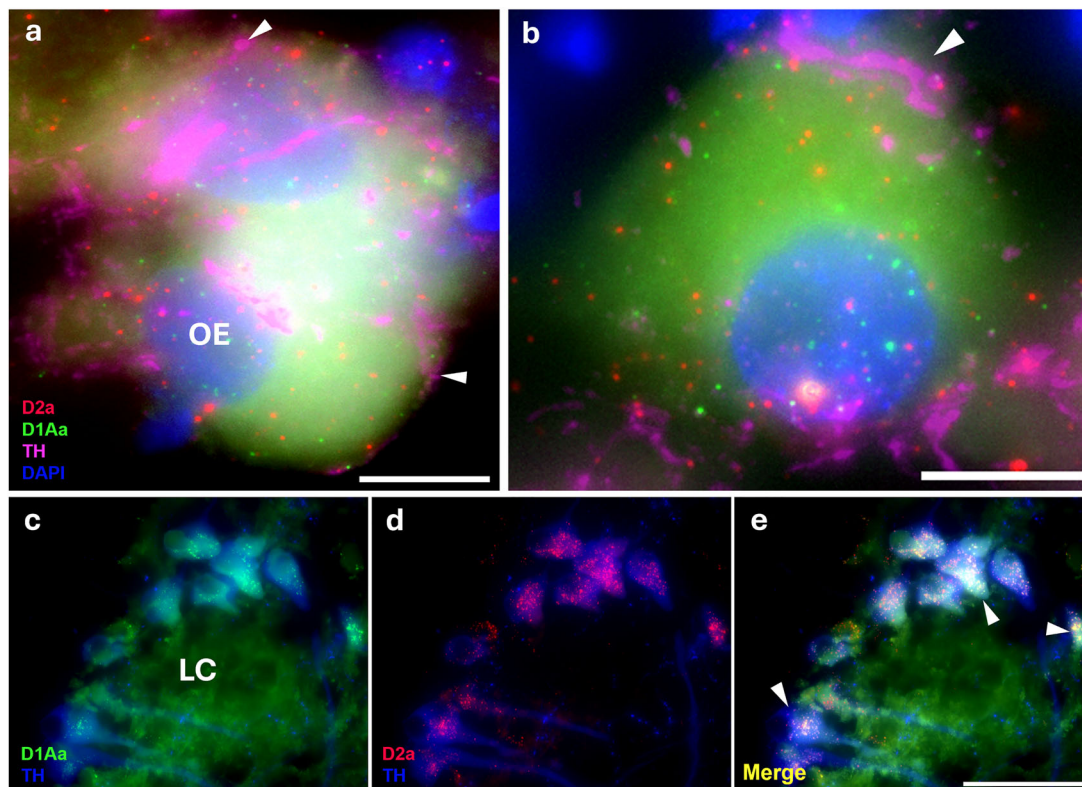


FIGURE 7 | *D1Aa* (green)/*D2a* (red) expression and TH-ir (magenta) innervation in hindbrain. (a and b) TH-ir fibers innervating/terminating at octavolateralis efferent nucleus (OE) cells co-expressing *D1Aa* and *D2a*. Arrowheads point to TH-ir fibers/putative terminals (c–e) Robust *D1Aa*/*D2a* expression in the noradrenergic locus coeruleus (LC). Arrowheads point to TH-ir LC cells in blue. Scale bars in a, b = 10 μ m; c, d, e = 50 μ m. DAPI, 4,6-diamidino-2-phenylindole; TH, tyrosine hydroxylase.

4.4 | *D1Aa* and *D2a* Expression in the Auditory Diencephalon and Midbrain

The TS receives catecholaminergic innervation, likely including both DA (from Tpp) and NA (from LC) (Adrio et al. 2002; Hornby and Piekut 1990; Perelmuter and Forlano 2015), and projects to other higher order auditory processing centers—PGL, AT, and CP (Bass et al. 2000). The TS in midshipman encodes concurrent male vocalizations (Bodnar and Bass 1997, 1999, 2001) and is thus a critical center for processing social auditory signals. Recent studies have shown excitatory and inhibitory DA modulation of auditory responses in the inferior colliculus (TS homolog) via D2-like receptors in rodents (Gittelman et al. 2013; Hoyt et al. 2019). Furthermore, in line with our findings, *D1* and *D2* receptor mRNA expression has been localized to the TS in the European eel (Kapsimali et al. 2000; Pasqualini et al. 2009) and the TS, CP, and AT of the African cichlid (O'Connell and Hofmann 2011). Furthermore, the CP similarly has a diverse response pattern to auditory stimuli but has broader tuning than neurons in the TS, suggesting that the CP may process more complex auditory stimuli (Lu and Fay 1995). Altogether, perhaps a robust distribution of both the *D1Aa* and *D2a* receptors is required in these auditory regions to efficiently process acoustic stimuli.

The TS also has reciprocal connections to the PAG, which has reciprocal connections with the Tpp and CP and receives input from AT (Kittelberger and Bass 2013). Although the PAG is

thought to be a vocal-auditory integration site, its connection to the Tpp provides direct anatomical evidence for incoming auditory information modulating the TH-ir cells in the Tpp (Forlano et al. 2017; Ghahramani et al. 2018; Vetter et al. 2025), which may, in turn, drive downstream changes in dopaminergic modulation of other auditory centers. Interestingly, a study in male midshipman found that dopamine injections in the PAG inhibit vocal production, and this inhibition could only be prevented by combined administration of D1 and D2 antagonists—D1/D2 antagonists administered individually had no effect (Allen et al. 2023). This highlights a unique, biologically necessary functional role for both D1 and D2 receptors within the PAG, and our results colocalizing *D1Aa*/*D2a* within individual PAG cells provide an anatomical framework for future mechanistic investigations. The high *D1Aa* and *D2a* expression in the PAG may be needed to serve its various roles in vocal-motor and auditory integration. A future comparative study in males and females may elucidate differential receptor patterns and potential functional roles within the various subdivisions of the PAG.

4.5 | *D1Aa* and *D2a* Expression in the Hindbrain

The cholinergic OE is a second nucleus in the brain that projects to the inner ear to modulate auditory sensitivity (Bass et al. 1994; Bleckmann et al. 1991; Tomchik and Lu 2005). Acetylcholine has an inhibitory effect on inner ear physiology in fish (Furukawa 1981). Our previous study found a robust summer increase in TH-

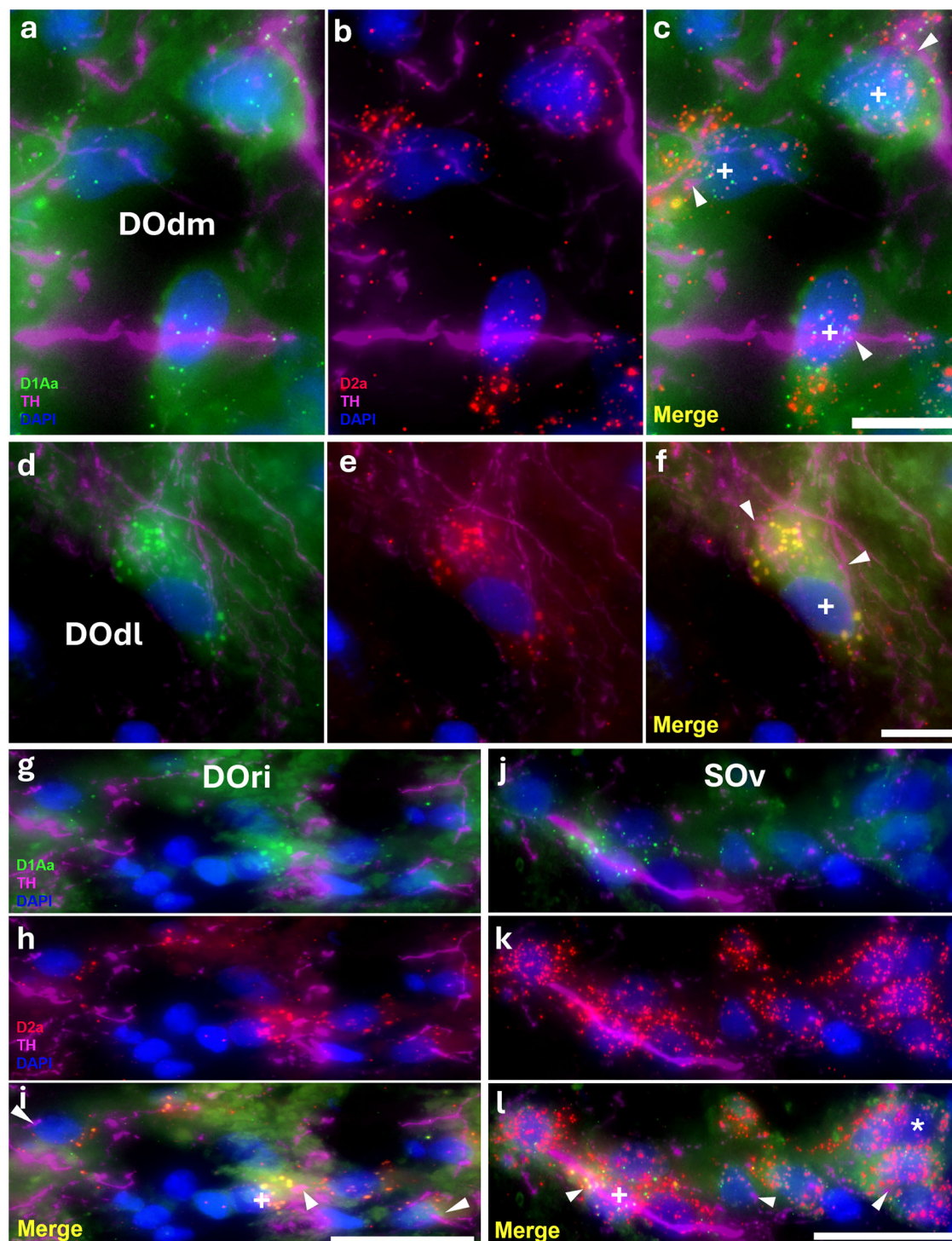


FIGURE 8 | Robust *D1Aa* (green)/*D2a* (red) expression and TH-ir (magenta) innervation of hindbrain auditory nuclei; nuclei stained with DAPI in blue. (a–i) TH-ir fibers innervating/terminating at dorsomedial (DOdm, a–c), dorsolateral (DOdl, d–f), and rostral intermediate (DORI, g–i) cells of the descending octaval nucleus (DO) co-expressing *D1Aa* and *D2a*. (j–l) TH-ir fibers innervating/terminating at ventral secondary octaval nucleus (SOv) cells expressing *D1Aa* and *D2a*. + labeled cells are *D1Aa*+/*D2a*+ and * labeled cells are *D1Aa*–/*D2a*+. All arrowheads point to TH-ir fibers/putative terminals. Scale bars in a–f = 10 μm; g–l = 25 μm. DAPI, 4,6-diamidino-2-phenylindole; TH, tyrosine hydroxylase.

ir innervation of the OE, and a subsequent tract-tracing study confirmed the source of TH-ir fibers is the dopaminergic cells in the Tpp (Forlano, Ghahramani, et al. 2015; Perelmutter and Forlano 2017). Thus, one would hypothesize that during the summer, reproductive months, higher dopamine innervation of

the OE may also be coupled with a high expression of inhibitory DARs, thus creating further inhibition of the cholinergic OE cells to enhance hearing sensitivity. However, our study reveals that the OE co-expresses both dopamine *D1Aa* and *D2a* receptors at roughly the same abundance, although we have no data on

the nonreproductive state. Based on the dense TH-ir innervation of OE neurons, we expected a higher density of *D2a* and/or *D1Aa* expression; therefore, it may be that one of the other 5 identified DARs predominates in this nucleus. Notably, the OE in the European eel also expresses both D1 and D2 receptors (Kapsimali et al. 2000; Pasqualini et al. 2009; Roberts et al. 1989). Previously documented seasonal changes, such as increases in BK channels and the density of sensory hair cells of the inner ear (Forlano et al. 2017; Rohmann et al. 2013), likely serve as longer term structural changes that broadly enhance hearing sensitivity and frequency encoding, whereas dopaminergic innervation of the OE (and inner ear) allows for a more context-dependent, complex central regulation of hearing at smaller time scales.

The noradrenergic LC receives dopaminergic input from the TPp (Kaslin and Panula 2001; Ma 1994) and projects widely to the midbrain and forebrain, which likely includes higher auditory areas (Tay et al. 2011; Perelmuter and Forlano 2015). Thus, seasonal changes in CA innervation of these regions may be reflected by a mix of DA and NA. Although there are no robust studies investigating NA modulation of central auditory circuits in teleost fishes, studies in rodents and birds have identified NA's important role in improving the signal-to-noise ratio of auditory responses and sharpening neuronal tuning in the auditory cortex (Gaucher and Edeline 2015; Ikeda et al. 2015; Yang et al. 2021). Interestingly, LC cells themselves robustly express both *D1Aa* and *D2a* receptor, suggesting that dopamine from the TPp may play a complex role in modulating LC function in addition to its widespread targets. Lastly, the co-expression of *D1Aa* and *D2a* in the descending and secondary octaval nuclei similarly suggests a broader pattern in which dopamine from the TPp may provide nuanced, region-specific regulation of auditory processing at multiple levels.

4.6 | Potential DAR Regulation by Steroids

Previous studies in midshipman have also shown that nonreproductive fish implanted with estrogen or testosterone mimic the peripheral auditory sensitivity of summer, reproductive fish (Sisneros, Forlano, Deitcher, et al. 2004). Furthermore, reproductive females have significantly less *D2a* receptor expression in the inner ear compared to reproductive females (Perelmuter et al. 2019) and nonreproductive females implanted with testosterone mimic some of the seasonal changes in catecholamine innervation of the auditory hindbrain, as well as the inner ear (Perelmuter et al. 2021). Importantly, androgen and estrogen receptors, along with aromatase (the enzyme that converts testosterone to estrogen), have been localized to the TPp (Forlano et al. 2005, 2010). Thus, steroid regulation of dopaminergic TPp neurons in midshipman is one likely mechanism to modulate auditory sensitivity and processing.

The seasonal surge in gonadal hormones, particularly testosterone, in pre-nesting female midshipman fish (see Sisneros, Forlano, Knapp, et al. 2004b) may act on androgen receptors on TPp neurons to regulate the expression of D2 autoreceptors, which could contribute to the downstream seasonal reorganization of dopamine innervation of other auditory centers. Testosterone has been shown to influence dopaminergic function and receptor

expression in many different organisms. In whiptail lizards, androgen implants in castrated males decrease *D1* and *D2* receptor expression in the nucleus accumbens (O'Connell et al. 2012). Adolescent syrian hamsters treated with anabolic/androgenic steroids increase dopamine and *D2* receptor expression in the anterior hypothalamus (Schwartz and Melloni 2010). In adolescent rats, androgens have been shown to increase *DAT*, vesicular monoamine transporter (*VMAT*), and *D2* receptor mRNAs in the substantia nigra as well as decrease *D3* mRNAs (Purves-Tyson et al. 2014); furthermore, non-aromatizable dihydrotestosterone mimicked the effect of testosterone, whereas estradiol did not, suggesting androgen-specific regulatory mechanisms (Purves-Tyson et al. 2014). It is also interesting to note that the effects of androgens on dopamine signaling in rats and hamsters are most robust in adolescence when gonadal hormone levels surge and dopaminergic circuits are still maturing. These findings support the hypothesis that surges in testosterone in pre-nesting female midshipman play a critical role in the robust seasonal reorganization of the dopamine circuit via TPp *D2a* regulation.

Another proposed mechanism by which steroids regulate dopamine signaling involves estrogen receptor-mediated transcriptional control of DAR expression. Estrogen binds to its receptors, which then dimerize and bind to estrogen response element (ERE), which can serve as transcription factors for specific genes. Previous studies show estrogen treatment in ovariectomized rats increasing *D1* and *D2* striatal DAR densities (Chavez et al. 2010; Falardeau and Di Paolo 1987; Lévesque and Di Paolo 1991). In nonhuman primates, *D1* and *D2* receptor availability fluctuates throughout the menstrual cycle, suggesting that endogenous estrogen levels may dynamically regulate DAR expression (Czoty et al. 2009). Ultimately, although literature supports the hypothesis that estrogen modulates DAR expression, the precise downstream mechanisms remain unclear.

4.7 | Functional Implications of D1/D2 Co-Expression

Robust co-expression of both *D1Aa* and *D2a* receptor transcripts suggests the downstream membrane protein receptors are in the same cell. Previous studies have shown that striatal spiny neurons in rodents co-express *D1/D2* (Aizman et al. 2000), and *D1A* and *D2* receptors are co-expressed in Area X neurons in songbirds (Kubikova et al. 2010). Interestingly, dopaminergic neurons that project to mushroom body neurons in fruit flies co-express the genes that encode *D1*-like and *D2*-like receptors and were found at the presynaptic site, suggesting a dual autoreceptor system that precisely modulates dopamine release via *D2* inhibition and *D1* positive feedback (Hiramatsu et al. 2025). This contrasts with the TPp in midshipman, where we only found *D2a*; however, the other two *D1*-like receptors (*D1Ab* and *D1Ba*) must be localized to rule out a dual autoregulatory role.

D1 and *D2* receptors also heavily differ in dynamics and affinity for dopamine. In vivo, the dopamine affinity of *D2Rs* has been reported to be ~100× greater than *D1Rs* (Beaulieu and Gainetdinov 2011; Costa and Schoenbaum 2022; Richfield et al. 1989). Thus, *D1Rs* require much larger dopamine concentrations for activation compared to *D2Rs*. Because of the low concentration of dopamine required to activate *D2Rs*, they are believed to be

activated by tonic release of dopamine and only deactivated by transient dips in dopamine, whereas D1Rs are believed to be activated by a phasic release of dopamine (Dreyer et al. 2010; Grace 2000; Missale et al. 1998). Importantly, at a baseline release of dopamine, D2Rs but not D1Rs are proposed to be activated (Costa and Schoenbaum 2022). We therefore hypothesize that neurons innervated by the TPp (or other DA source) that contain both D1Rs and D2Rs have a baseline level of inhibition through D2Rs and dopaminergic cells in the TPp may either pause firing to release inhibition of the target cell or burst fire to excite the target cell. This multilayered regulatory mechanism may be necessary for fine-tuning neuromodulation and processing complex auditory signals, including discerning reproductive mating calls.

Although heavily debated, it has been proposed that, despite D1Rs typically being coupled to Gs proteins while D2Rs are typically coupled to Gi proteins (see Section 2), when these receptors are activated together, they can form a hetero-oligomer complex that couples to the Gq protein (Hasbi et al. 2020; Perreault et al. 2011; Rashid et al. 2007). These Gq proteins then activate phospholipase C (PLC), which catalyzes the breakdown of phosphatidylinositol phosphate (PIP) into diacylglycerol (DAG) and inositol trisphosphate (IP3), leading to protein kinase C (PKC) activation and then calcium release (Mizuno and Itoh 2009). The existence of D1–D2 hetero-oligomers has not come without criticism, however (see Frederick et al. 2015). Considering the robust co-expression of *D1Aa* and *D2a* throughout the central auditory system in midshipman, these receptors forming hetero-oligomers are certainly a promising hypothesis that may explain the functional purpose of co-expression. Altogether, these may represent complex mechanisms by which dopamine can act to produce its effects, and further studies are needed to delineate which mechanisms are most relevant for modulating auditory function.

5 | Conclusions

Here, we provide a comprehensive characterization of dopamine *D1Aa* and *D2a* receptor transcripts across the central auditory system of the plainfin midshipman fish, an important model for investigating neural mechanisms of vocal-acoustic communication in vertebrates. The TH-ir neurons of the TPp—the dopaminergic source to various auditory centers that is partly responsible for the seasonal reorganization of catecholamine innervation—exhibit very dense *D2a* expression while lacking *D1Aa*, suggesting robust autoregulatory control at both somatodendritic and axon-terminal sites. Nearly all auditory nuclei downstream of the dopaminergic TPp projections contain populations of neurons that either co-express *D1Aa* and *D2a* or express *D2a* exclusively, suggesting diverse, complex, and region-specific dopaminergic modulation. The previously documented expression of androgen and estrogen receptors within the TPp coupled with prior evidence from teleost and mammalian models demonstrating androgen regulation of dopamine synthesis and receptor expression supports the hypothesis that seasonal fluctuations in gonadal steroids or other seasonally regulated hormones may regulate DAR expression, particularly within the TPp. However, further studies are needed to confirm the exact mechanism.

Ultimately, these findings reveal a complex dopaminergic cytoarchitecture throughout the midshipman central auditory system. These results lay the neuroanatomical groundwork for future investigations into reproductive state-dependent changes in DAR expression, functional implications of D1/D2 co-expression, and steroid hormone regulation of dopaminergic signaling pathways. Given the evolutionary conservation of central auditory processing centers across vertebrates that use acoustic communication, these findings provide key comparative insights into dopaminergic modulation of auditory function.

Acknowledgments

We thank Jonathan Perelmuter for his contributions to the methodology of the FISH-IHC and Margaret Marchaterre for help with the initial pilot ISH. We also thank Sofia Gray and Joseph Sisneros for animal collections. This research was supported by the Macaulay Goldsmith Scholars Fellowship, Leonard & Claire Tow Undergraduate Research Program, NSF REU in Psychology and Neuroscience [2349686], and CUNYBA Thomas W. Smith Fellowship to Kobi Kobi. This study is based upon work by Paul M. Forlano supported while serving at the National Science Foundation.

Funding

This research was supported by the National Institutes of Health [1R15DC020327] and PSC-CUNY [68877-00 46] to Paul M. Forlano.

Ethics Statement

All procedures involving animals were approved by the Institutional Animal Care and Use Committee (IACUC) at Brooklyn College.

Consent

The authors have nothing to report.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data supporting the findings of this study are available from the corresponding author upon reasonable request.

Peer Review

For transparency, the peer review documents associated with this article are available at <https://doi.org/10.1002/cne.70166>.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Supporting Figure S1: Robust *D2a* (red) expression is seen in the supracommissural nucleus of area ventralis of the telencephalon (Vs), but *D1Aa* (green) signal is not convincingly above background in this area; nuclei stained with DAPI in blue. Scale bar = 50 µm.