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Oxytocin-like receptor expression in evolutionarily conserved nodes of a vocal network associated with male courtship in a teleost fish

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Abstract

Neuropeptides, including oxytocin-like peptides, are a conserved group of hormones that regulate a wide range of social behaviors, including vocal communication. In the current study, we evaluate whether putative brain sites for the actions of isotocin (IT), the oxytocin (OT) homolog of teleost fishes are associated with vocal courtship and circuitry in the plainfin midshipman fish (*Porichthys notatus*). During the breeding season, nesting males produce advertisement calls known as "hums" to acoustically court females at night and attract them to nests. We first identify IT receptor (ITR) mRNA in evolutionarily conserved regions of the forebrain preoptic area (POA), anterior hypothalamus (AH), and midbrain periaqueductal gray (PAG), and in two topographically separate populations within the hindbrain vocal pattern generator– duration-coding vocal prepacemaker (VPP) and amplitude-coding vocal motor nuclei (VMN) that also innervate vocal muscles. We also verify that ITR expression overlaps known distribution sites of OT-like immunoreactive fibers. Next, using phosphorylated ribosomal subunit 6 (pS6) as a marker for activated neurons, we demonstrate that ITR-containing neurons in the anterior parvocellular POA, AH, PAG, VPP, and VMN are activated in humming males. Posterior parvocellular and magno/gigantocellular divisions of the POA remain constitutively active in nonhumming males that are also in a reproductive state. Together with prior studies of midshipman fish and other vertebrates, our

Abbreviations: ac, anterior commissure; AH, anterior hypothalamus; AT, anterior tuberal nucleus of hypothalamus; Cc, cerebellar crest; Cg, granule cell layer of the corpus of the cerebellum; Cm, molecular layer of the cerebellum; CP, central posterior nucleus; Cpost, posterior commissure; D, area dorsalis of telencephalon; Dc, central zone of D; Dd, dorsal zone of D; DIL, diffuse nucleus of the inferior lobe; DiV, diencephalic ventricle; Dl, lateral zone of D; Dm, medial zone of D; Dm-p, posterior division of Dm; DOdm, dorsomedial division of descending octaval nucleus; DOri/I, rostral intermediate/intermediate division of descending octaval nucleus; DPo, dorsal posterior nucleus of the thalamus; ECL, external cellular layer of the olfactory bulb; GL, glomerular layer of olfactory bulb; Had, dorsal division of the habenula; Hav, ventral division of the habenula; Hb, habenula; Hd, dorsal zone, periventricular hypothalamus; HoCo, horizontal commissure; Hv, ventral zone, periventricular hypothalamus; HV, hindbrain ventricle; IP, isthmal paraventricular nucleus; Is, isthmal auditory-vocal nucleus; LH, lateral hypothalamus; ll, lateral lemniscus; MED, cell plate of medial octavolateralis nucleus; MLF, medial longitudinal fasciculus; MV, midbrain ventricle; nLL, nucleus of the lateral lemniscus; OB, olfactory bulb; OEN, octaval efferent nucleus; On, olfactory nerve; Opn, optic nerve; PAG, periaqueductal gray; PGl, lateral division of nucleus preglomerulosus; PGm, medial division of nucleus preglomerulosus; PGZ, periventricular gray zone; PHT, preoptico-hypophysial tract; PL, paralemniscal midbrain tegmentum; PM, magnocellular nucleus of the POA; PMg, gigantocellular neurons of PM; PMm, magnocellular neurons of PM; POA, preoptic area; PPa, anterior parvocellular nucleus of the POA; PPp, posterior parvocellular nucleus of the POA; PPv/d, periventricular pretectal nucleus, ventral and dorsal divisions; RF, reticular formation; SAC, stratum album centrale; SCN, suprachiasmatic nucleus; SD, saccus dorsalis; SGC, stratum griseum centrale; SGN, secondary gustatory nucleus; SOv, ventral division of secondary octaval nucleus; SPV, stratum periventriculare; SV, saccus vasculosus; TeM, mesencephalic tectum; TL, torus longitudinalis; TLat, torus lateralis; TP, posterior tuberal nucleus; TPp, periventricular nucleus of the posterior tuberculum; TS, torus semicircularis; TSd, deep layer of TS; TSp, periventricular layer of TS; V, area ventralis of the telencephalon; Vc, central nucleus of V; Vd, dorsal nucleus of V; Vg, granule cell layer of the valvula; Vi, intermediate nucleus of V; VIIm, facial motor nucleus; Vm, molecular layer of the valvula; VMN, vocal motor nucleus; Vp, postcommissural nucleus of V; VPN, vocal pacemaker nucleus; VPP, vocal prepacemaker nucleus; Vs, supracommissural nucleus of V; vT, ventral tuberal nucleus of hypothalamus; Vv, ventral nucleus of V; XL, vagal lobe; Xmn, vagal motor nucleus

findings suggest that IT-signaling influences male courtship behavior, in part, by acting on brain regions that broadly influence behavioral state (POA) as well as the initiation (POA and PAG) and temporal structure (VPP and VMN) of advertisement hums.

KEYWORDS
isotocin receptor, oxytocin, teleost fish, vocal communication, vocal motor network

1 | INTRODUCTION

Communication is important for navigating social encounters and is essential to reproductive success and survival in many species across a broad range of vertebrate groups. One way that the nervous system can adapt to support novel forms of communication, including social context-dependent vocalization, is through the evolutionary modification of ancient hormone signaling systems within the brain (Adkins-Regan, 2013; Feng & Bass, 2017; Hoke et al., 2019; Katz & Lillvis, 2014). Although much of this research has focused on steroid hormone action (Fuxjager & Schuppe, 2018; McGlothlin & Ketterson, 2008), neuropeptides have also been strongly implicated in mechanisms underlying adaptive plasticity in communication function, including vocalization (Goodson, 2011, 2013; Goodson & Bass, 2001; Insel & Young, 2000; Theofanopoulou et al., 2017)

Nonapeptides, including oxytocin (OT) and its homologues (Donaldson & Young, 2008; Venkatesh & Brenner, 1996), modulate diverse aggressive, reproductive, and affiliative behaviors (reviewed in Adkins-Regan, 2013; Goodson, 2013; Insel, 2010; Theofanopoulou et al., 2017). Given the central role of social communication in many agonistic and reproductive contexts, it is not surprising that investigations across vertebrate lineages have implicated this highly conserved family of peptides in vocal and, more broadly, social functions. Several studies in mice document that OT is necessary for innate vocal ability (Takayanagi et al., 2005; Winslow et al., 2000). In oscine songbirds, the OT homolog mesotocin (also the OT homolog in amphibians and reptiles) modulates aspects of song used for female courtship (Pedersen & Tomaszycki, 2012; but see Goodson et al., 2004). As with all OT-like peptides, mesotocin exerts its physiological and behavioral effects through a G-protein-coupled receptor known as the mesotocin receptor (oxytocin receptor homolog; Theofanopoulou et al., 2021). In songbirds, mesotocin receptors are not only present in all neural compartments specific to song learning (Leung et al., 2011), but also expressed in highly conserved nodes within the vocal control network of the forebrain and midbrain (see Goodson et al., 2003). These findings suggest that OT and OT-like peptides may act on neurons in regions that directly modulate vocal output. Nonetheless, it remains unclear whether neurons that express OT-like receptors are targets of OT-like actions that regulate, for example, the performance of vocal courtship.

We use a highly vocal teleost fish, the plainfin midshipman (*Porichthys notatus*), to test the hypothesis that OT-like signaling in the brain is associated with vocal behavior. These fish provide an ideal opportunity to identify brain sites where isotocin (IT), the OT-homolog in teleost fish (Donaldson & Young, 2008), acts to regulate vocal courtship. During the late spring and summer breeding season, nesting males reliably produce advertisement "hums" to acoustically court females at night and attract them to their nests (Figure 1a). Each hum can last up to two hours and is produced repeatedly throughout an evening of courtship by contracting swim bladder muscles at ≈100 Hz (at ≈16°C ambient temperature). Vocal output is controlled by a well-studied central network (Figure 1b; e.g., see Chagnaud et al., 2011; Goodson & Bass, 2002). Importantly, neuroanatomical and neurophysiological data suggest that vocal network nuclei in the midshipman brain are functionally comparable to neuronal populations controlling vocal behaviors across tetrapods (Bass, 2014). Thus, studying where IT can act within the vocal network of midshipman provides an evolutionarily conserved framework for establishing the influence of OT-like peptides on vocalization (Figure 1b).

As in other teleosts (Holmqvist & Ekström, 1991; Huffman et al., 2012; van den Dungen et al., 1982), there is ample neuroanatomical evidence describing where OT-like somata and fibers are located in the midshipman brain, including the vocal network (Foran & Bass, 1998; Goodson & Bass, 2000c; Goodson et al., 2003). Neurophysiological and transcriptomic studies support an active role for IT-signaling within this network (Goodson & Bass, 2000a; Tripp et al., 2018). Transcriptome studies further show that IT receptor (ITR) expression changes in the preoptic area (POA) and contiguous anterior hypothalamus (AH) across reproductive-related behavioral tactics (Tripp et al., 2018). Yet, little is known regarding the targets of forebrain IT neurons, and more specifically their role in vocalization.

Here, we identify sites of IT action associated with vocal courtship in male midshipman fish. We first investigate the distribution of ITR, with particular attention to central vocal, auditory, and neuroendocrine (POA-AH) nuclei (see Figure 1b). Although a prior study of midshipman brain maps the distribution of putative IT-synthesizing somata and their processes using an OT antibody (Goodson et al., 2003), we repeat these experiments to verify whether such labeling overlaps locations of ITR expression. Next, we use a marker for activated neurons, phosphorylated ribosomal subunit 6 (pS6; Knight et al., 2012), to determine whether vocal network regions rich in ITR are activated during courtship humming. Given past findings (see above), we hypothesize that nuclei within evolutionarily conserved vocal forebrain, midbrain, and hindbrain regions (Figure 1b) exhibit robust ITR expression associated with vocal courtship-related neural activity and OT-like immunoreactivity.

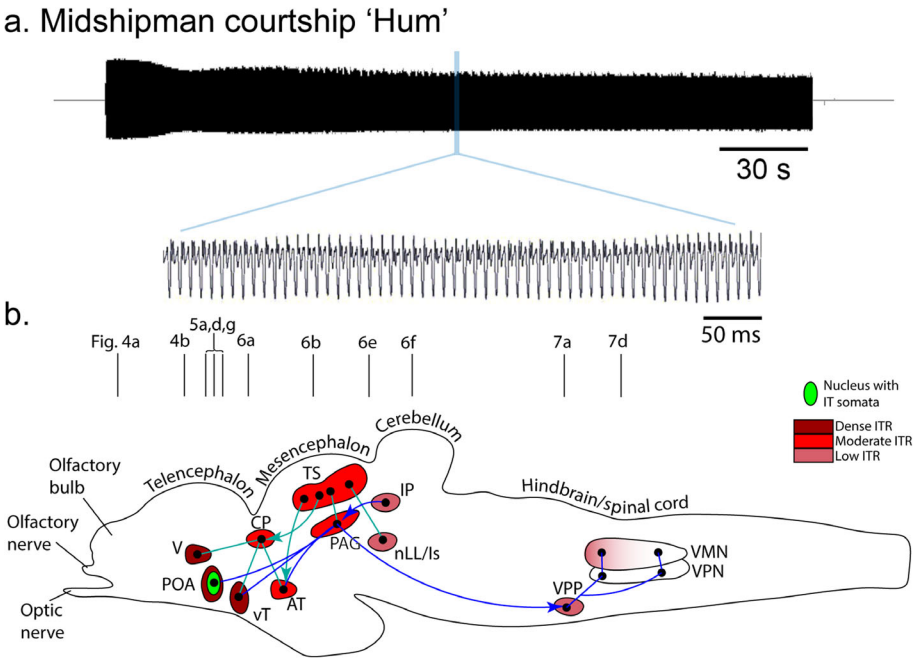

FIGURE 1 Vocal behavior and vocal-acoustic network of plainfin midshipman fish (*Porichthys notatus*). (a) Oscillogram records on two timescales of the courtship "hum" produced by type I males during the breeding season. (b) Line drawing in sagittal plane of midshipman brain depicting major vocal and auditory regions (modified from Goodson & Bass, 2002; Bass & McKibben, 2003; Kittelberger et al., 2006; Pengra et al., 2018). Blue lines show connections of vocal motor system and teal lines connections of auditory system. Vertical lines indicate approximate levels of low magnification images in Figures 3–6. Vocal nuclei are shaded in various shades of red to denote the relative amount of isotocin receptor (ITR) mRNA present in that region (see legend). A filled green circle denotes where putative IT somata, detected with an oxytocin antibody, are present in the preoptic area (POA). Other abbreviations: AT, anterior tuberal nucleus; CP, central posterior nucleus; IP, isthmal paraventricular nucleus; Is, isthmal auditory-vocal nucleus; nLL, nucleus of the lateral lemniscus; PAG, periaqueductal gray; TS, torus semicircularis; V, area ventralis of the telencephalon; VMN, vocal motor nucleus; VPN, vocal pacemaker neurons; VPP, vocal prepacemaker neurons; vT, ventral tuberal nucleus

2 | MATERIALS AND METHODS

2.1 | Animals

The plainfin midshipman has two male reproductive morphs with distinct developmental histories and reproductive tactics (Bass, 1996). Type I males broadcast advertisement hums (Figure 1a) from nests that they build under rocky shelters, provide sole parental care for eggs that line the walls of the nest, and aggressively defend their nest against intruders (Brantley & Bass, 1994; Tripp & Bass, 2020). We focus on type I males as they have the most diverse vocal repertoire, are the only morph that produce courtship hums, and are found in abundance during the breeding season (Bass, 1996; Brantley & Bass, 1994; McIver et al., 2014). Type I males (n = 16; standard length, 14.6–21.1 cm; body mass, 38–103 g) were hand-collected from nests in northern California during the breeding season, between May and August of 2019. These animals were used for either neuroanatomical chartings of ITR and IT (n = 7) or in behavioral experiments (n = 9; see Section 2.2 for more details). Fish were maintained overnight at the University of California, Davis Bodega Marine Laboratory and then shipped to Cornell University where they were housed in environmental control rooms in 25 gal, artificial seawater tanks at 16–18°C on a 15-h:9-h light: dark cycle as to mimic natural long summer days. Males were provided artificial nests and housed individually; past work has shown that under these conditions, type I males reliably produce courtship hums (Brantley & Bass, 1994; Feng & Bass, 2016).

2.2 | Experimental design

After type I males were acclimated to aquaria, we used hydrophones (Aquarian Audio H1a) as previously described to monitor vocal behavior (Feng & Bass, 2016; Tripp et al., 2020). Observers remotely monitored vocal courtship behavior and removed fish 15 min after the onset of humming. Fish were then immediately isolated in 5 gal, opaque buckets for 120 min during which time there was no humming. This timepoint was chosen following a pilot study that assessed the time-course of pS6 protein expression in the midshipman vocal network (Tripp et al., 2020). Silent (nonvocal) controls were also removed from their home aquarium during the dark period and isolated for the same time period (we did not hear any grunts or growl, which are easily audible, from these males for 2 h prior to isolation). We confirmed that there was no significant difference between control (n = 5) animals and hummers (n = 4) in body size (standard length, $p > 0.2$; mass, $p > 0.2$).

2.3 | Tissue collection and preparation

Fish were deeply anesthetized for 5 min in 0.025% benzocaine dissolved in aquarium water, ex-sanguinated, and then perfused transcardially with ice-cold marine teleost Ringers solution (see http://comm.archive.mbl.edu/BiologicalBulletin/COMPENDIUM/CompTab6.html#TAB6B-F), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Brains were immediately dissected, postfixed in 4% PFA for 1 h at room temperature, and then washed in 0.1 M PB at 4°C overnight. The next day, the tissue was cryopreserved in 30% sucrose in 0.1 M PB and embedded Tissue-Tek O.C.T. compound (Sakura Finetek, Torrence, CA, USA) before being stored at −80°C. Brains were sectioned at −20°C in a cryostat (Hacker Instruments and Industries Inc., Winnsboro, SC, USA) into 25 μm transverse sections and then thaw-mounted onto Superfrost Plus slides (ThermoFisher Scientific, Waltham, MA, USA). Adjacent sections were collected as three complete series through the brain. Tissue sections were then allowed to dry at room temperature and later stored at −80°C. All procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

2.4 | Cloning and partial sequencing of the isotocin receptor

To identify the ITR sequence in midshipman, we generated degenerate PCR primers (forward primer: TGCTCCTCTGRCTACTWGTGC; reverse primer: AGCCTKGGGGARCYAAAGCT) from ~ 567 bp region of this gene that was highly conserved across teleost fishes. Each PCR reaction contained 100 ng of cDNA (from brain lysate from a mixture of types I and II males), 0.5 μM of forward primer, 0.5 μM of reverse primers, iProof polymerase, 10 μM DNTPs, and reaction buffer. Reactions were then run at 98°C for 30 s, followed by 30 cycles of 98°C for 10 s; 55°C for 10 s; and 72°C for 30 s with a final extension step at 72°C for 5 min. The amplified sequence was then imaged on 1% agarose gel to confirm band size. Bands were then excised from the gel and purified using a PCR purification kit (Qiagen).

The purified product was inserted into pCRII-blunt TOPO vector and transformed into TOP10 competent cells via heat shock for 30 s at 42°C. Cells were then cultured in SOC media for 1 h at 37°C before being spread on LB agar plates with 50 mg/ml kanamycin. The next day, individual colonies were transferred to LB liquid culture containing kanamycin, allowed to grow overnight, and then purified by using a miniprep kit (Qiagen). The resulting minipreps were sent to the Cornell Institute of Biotechnology for sequencing to verify the identity and orientation of PCR product in each plasmid.

2.5 | Riboprobe synthesis

Plasmids were linearized with EcoRV restriction enzymes. In vitro transcription of antisense probes was carried out in a reaction containing 0.1 M DTT, 2.0 μl transcription buffer, RNase inhibitor, SP6 Polymerase, 10× Dig Labeling Mix (40 U/μl), and the 1 μg of linearized midshipman ITR plasmid. Probes were then precipitated overnight in isopropyl alcohol and lithium chloride at −20°C, centrifuged at 14,000 *g* for 30 min, and reconstituted in 90% formamide in nuclease free water.

2.6 | Single immunofluorescence

First, slides were incubated in blocking solution (0.2% bovine serum albumin (BSA, Sigma Aldrich, St. Louis, MO, USA), 0.3% Triton-X 100 (Sigma Aldrich), and 10% normal goat serum (NGS, ThermoFisher) in phosphate buffered saline (PBS). Sections were then incubated overnight in rabbit anti-OT (Millipore, AB911; see Table 1) in blocking solution at room temperature in a humidified chamber. Following primary antibody incubation, slides were washed three times in PBS for 10 min each, and then incubated 2 h in Alexafluor 488 (1:500) in 10% NGS. After secondary antibody incubation, slides were washed in PBS three times for 10 min each, and then slides were cover slipped with ProLong Gold with DAPI (ThermoFisher). After coverslipping, slides were allowed to dry at room temperature overnight, and edges were then sealed with nail polish. Slides were stored at 4°C.

2.7 | Antibody characterization

Previous studies have validated the efficacy of this antibody for midshipman; preadsorption with 50 or 100 μM of the IT peptide for 4 h at room temperature abolished all IT staining (Tripp & Bass, 2020). Since there was no observable staining, this control confirmed that immunostaining is likely specific to IT (Tripp & Bass, 2020). The pS6 antibody has also been validated in midshipman and another teleost (Butler et al., 2018; Tripp et al., 2020). First, this antibody was characterized through a western blot that showed a single band of 32 kDa MW. This work also identified intense pS6 labeling in motoneurons within the vocal motor nucleus (VMN) of humming animals, which was absent in animals that did not produce vocalizations. These experiments also concluded that 120 min after the performance of a behavior was an optimal time window to observe pS6 signal in the VMN. The role of VMN motoneurons in vocal behavior has been extensively documented through neurophysiology and tract tracing experiments, including their direct innervation of the paired vocal muscles attached to the swim bladder (see Chagnaud et al., 2012). In aggregate, these controls confirm pS6 as a reliable neural activity marker for the VMN and other neuronal populations underlying vocal behavior in midshipman fish. In the current study, we also performed controls leaving out either the IT or pS6 primary antibodies. These eliminated IT or pS6 staining, respectively.

2.8 | In situ hybridization

Brain sections (n = 7) were first fixed in 4% paraformaldehyde for 5 min and then washed in PBS twice for 3 min each. Sections were then acetylated for 10 min in a 0.1 M TEA solution with 0.33% acetic

TABLE 1 List of antibodies used in this study

Antibody name	Immunogen	Host	Antibody type	Dilution	Manufacturer	Catalogue number, RRID
Primary antibodies						
Oxytocin	Synthetic oxytocin conjugated to thyroglobulin	Rabbit	Polyclonal	1:500	Millipore	AB911, RRID:AB_2157629
Phospho-S6 ribosomal protein (Ser235/236)	Gamma immunoglobin	Rabbit	Monoclonal	1:250	Cell-signaling	Cat# 4858, RRID:AB_916156
Secondary antibody						
Donkey antirabbit Alexa fluor 488	Gamma immunoglobin	Donkey	Polyclonal	1:500	ThermoFisher Scientific	Cat# A-21206, RRID:AB_2535792
Antidigoxigenin-AP, fab fragments	Gamma immunoglobin	Sheep	Polyclonal	1:500	Roche	Cat# 11093274910, RRID:AB_2734716

anhydride, rinsed once with PBS, and then serially dehydrated in 70%, 95%, and 100% ethanol. Sections were next incubated in hybridization buffer (300 mM NaCl, 20 mM Tris-HCl pH 8, 5 mM EDTA, 10 mM Na_2HPO_4 (pH = 7.2), 10% dextran sulfate, 1X Denhardt's, 500 μg/ml tRNA, 200 μg/ml Herring sperm DNA, 50% formamide in deionization water) at room temperature for 1 h. Slides were then transferred to hybridization buffer with either a sense or antisense DIG-labeled riboprobe. Slides were coverslipped, sealed, and incubated overnight at 65°C. The following day, sections were first rinsed and coverslips removed, and then washed five times in 5X SSC for 10 min, followed by four times in 0.2X SSC for 30 min. Sections were next rinsed briefly in 100 mM Tris, pH 7.5; 150 mM NaCl, and then blocked for 60 min at room temperature in blocking buffer (0.1 M Tris, 150 mM NaCl, 10% normal horse serum). Slides were then transferred to blocking buffer with an alkaline phosphatase conjugated anti-DIG antibody (Roche) and allowed to incubate at 4°C overnight. Next, the slides were washed three times for 10 min in 100 mM Tris, pH 7.5; 150 mM NaCl. The slides were then developed by incubating with SIGMAFAST™ Fast Red TR/Naphthol AS-MX Tablets (Sigma Aldrich) reconstituted in 100 mM Tris-HCl (pH = 8.0) for 2 h. The slides were then washed three times with PBS for 5 min, mounted with Prolong Gold with DAPI, and then coverslipped. Finally, edges were sealed with nail polish and let sit overnight at room temperature.

2.9 | Double in situ hybridization and immunofluorescence

To test whether ITR-expressing neurons were activated in different brain regions of humming males, we performed double in situ hybridization (for ITR) and immunohistochemistry (for the neural activation marker pS6; $n = 9$). The in situ portion of this protocol was identical to that used to detect ITR mRNA alone (Section 2.7). Following the final PBS washes in the in situ hybridization protocol, slides from humming or control, nonhumming animals were processed for immunofluorescence. First, slides were incubated for 1 h in blocking solution (0.2% bovine serum albumen, BSA, Sigma Aldrich), 0.3% Triton-X 100 (Sigma-Aldrich) and 10% NGS (ThermoFisher; in PBS). Then, sections were incubated overnight in rabbit anti-pS6 (Ser235/236; Cell Signaling Technology, Cat# 4858; see Table 1) blocking solution at room temperature in a humidified chamber. Following primary antibody incubation, slides were washed three times in PBS, then incubated for 2 h in Alexafluor 488 PBS + 10% NGS. After secondary antibody incubation, slides were washed in PBS three times for 10 min, followed by one 10 min wash in double distilled water, and then coverslipped with ProLong Gold with DAPI (ThermoFisher). After coverslipping, slides were allowed to dry at room temperature overnight; then, edges were sealed with nail polish. Slides were stored at 4°C.

In a subset of humming animals ($n = 3$), we verified that the in situ protocol did not alter pS6 expression ($p > 0.2$). Namely, we counted the number of pS6+ neurons in the periaqueductal gray (PAG) and VMN on adjacent sections that were only processed for immunofluorescence.

2.10 | Image acquisition and analysis

All staining was initially evaluated on a Zeiss Axioskop 2 plus microscope. Images of representative sections were collected using a Zeiss LSM 880 using a 20× lens confocal microscope with Zen software (v2.1). To acquire representative coronal brain sections, we took multiple tiles (20× images) of the ITR mRNA (561 laser) and DAPI (405 laser) signal and stitched them together using Zen. For each region of interest, we acquired a z-stack at 4 μm intervals of ITR mRNA, pS6 (488 laser), and DAPI signals. An individual blind to the treatment groups (E.R.S.) performed all quantitative assessment of labeled sections. The researcher counted all ITR positive cells that also expressed pS6. In each region of interest, the researcher performed these counts on two adjacent sections.

For neuroanatomical chartings of ITR, two researchers (E.R.S and A.H.B.) independently classified expression in nuclei within the midshipman brain as: light (observable ITR mRNA expression, but low number of stained cells in an identified brain region), moderate (more than half the neurons in a region expressing ITR mRNA, with some cells expressing multiple puncta per cell), or dense (nearly all cells within a region expressing ITR mRNA, with many cells expressing multiple puncta per cell). We used similar criteria for IT fibers; however, instead

TABLE 2 Species and accession numbers for sequences for oxytocin-like receptor multiple sequence alignment and gene tree production

Species	Accession number
Danio rerio	NM_001199370.1
Trematomus bernacchii	XM_034138630.1
Haplochromis burtoni	XM_005941093.3
Thalassophryne amazonica	XM_034166612.1
Rana catesbeiana	AY277925.1
Anolis carolinensis	XM_003224891.3
Taeniopygia guttata	XM_002188266.4
Mus musculus	NM_001081147.2
Homo sapiens	X64878.1

of mRNA puncta, we classified staining based on fiber density. Expression patterns were verified across multiple animals ($n = 7$).

2.11 | Multiple sequence alignment and phylogenetic analysis

Species differences in the OT-like receptor sequences were analyzed using Multiple Sequence Comparison by Log-Expectation (MUSCLE; Table 2; https://www.ebi.ac.uk/Tools/msa/muscle/). Sequences were aligned using ClustalW setting in MUSCLE. Differences in OT-like receptor sequences were then visualized using JalView (version 2.11.4).

2.12 | Statistical analyses

All analyses were performed using R. Data were (log [1 + x]) transformed to achieve normality, as Q–Q plots and Shapiro–Wilk tests indicated that these transformations yielded more normally distributed data. Independent sample *t*-tests were performed to assess condition differences (humming $n = 4$; non-humming $n = 5$) in the percent of pS6+ cells that also express ITR mRNA across vocal network populations. These tests were followed by using Benjamini–Hochberg corrections to account for multiple contrasts using the "padjust" function in R.

3 | RESULTS

3.1 | Overview of ITR and IT expression

We generated a 567 bp probe that encompassed most of the coding region of the midshipman *ITR* gene. Midshipman are members of a single order (Batrachoidiformes) and family (Batrachoididae) often referred to as toadfishes (Hubbs & Nelson, 1978). The region that we cloned exhibited strong conservation with another batrachoidid (*Thalassophryne amazonica*, 94%), but less conservation with more distantly related teleosts (77%; e.g., *Tetraodon nigroviridis*) to 82%; e.g., *Toxotes jaculatrix*), birds, or mammals (see Figure 2a,b). Using this RNA probe, we mapped the distribution of ITR throughout the brain of type I males in reproductive condition (see Section 2).

Expression of ITR mRNA, as visualized through in situ hybridization, resembled oxytocin receptor labeling in mouse brain (Maldonado et al., 2021; Young & Song, 2020); namely puncta-like expressions largely expressed within cell nuclei or cytoplasm. The use of another marker, pS6, allowed us to occasionally visualize ITR mRNA in pS6 labeled processes (see below for more details). Conversely, slides hybridized with the sense probe did not show any staining.

We observed strong ITR labeling in vocal, auditory, and neuroendocrine regions of the brain that are part of the known central vocal network (Figure 1b), and in sensory areas that, to date, have not been considered as part of this network (see below). To identify sites of IT synthesis and determine whether labeled somata and fibers are found in locations where ITR is present, we performed immunofluorescence on adjacent sections using an OT antibody that was previously validated for midshipman (Tripp & Bass, 2020). Consistent with previous reports in midshipman (Goodson et al., 2003), we found that IT is mainly synthesized in forebrain POA neurons. However, OT-like fibers were often located in and around brain areas that exhibit ITR staining.

A complete summary of brain regions that exhibit ITR and OT-like, hereafter referred to as putative IT, somata and fibers can be found in Figure 3. The representative Nissl-stained, transverse sections at comparable levels in Figure 3 match the ones in Figure 1b of Tripp and Bass (2020), except for 3a and 3h which are from the same nonexperimental brain series. Figure 1a indicates the approximate levels of representative transverse sections along the rostral-caudal axis in Figures 4–7. The neuroanatomical nomenclature follows that used in prior publications on midshipman fish (e.g., Tripp & Bass, 2020) and other teleosts (Braford & Northcutt, 1983; Nieuwenhuys, 1963). The nomenclature used for the vasotocin-oxytocin family of peptides follows that used in earlier publications from this laboratory and others (e.g., Donaldson & Young, 2008), although we recognize the recent proposal that a single nomenclature using oxytocin can be used for all vertebrate phylogenies (Kelly & Goodson, 2014; Theofanopoulou et al., 2021).

3.2 | ITR and IT in olfactory bulb, telencephalon, and preoptic area

Isotocin receptor mRNA expression was moderate to light in the olfactory bulb, largely within the internal cell layer (ICL; red, Figures 3a and 4a,b). Receptor expression was also moderate to light in multiple subregions of the pallium, area dorsalis of the telencephalon (D). In the medial zone, Dm (Figures 3b–e, 4c and 5a,ai,d,g), ITR had a layered-like appearance over somata near the ependymal surface that was more apparent posterior to the anterior commissure (Dm-p, Figures 3c–e and 5ai). Although boundaries between dorsal (Dd) and lateral (Dl) zones of the pallium are the most difficult to distinguish in midshipman brain, ITR expression occurred throughout Dd

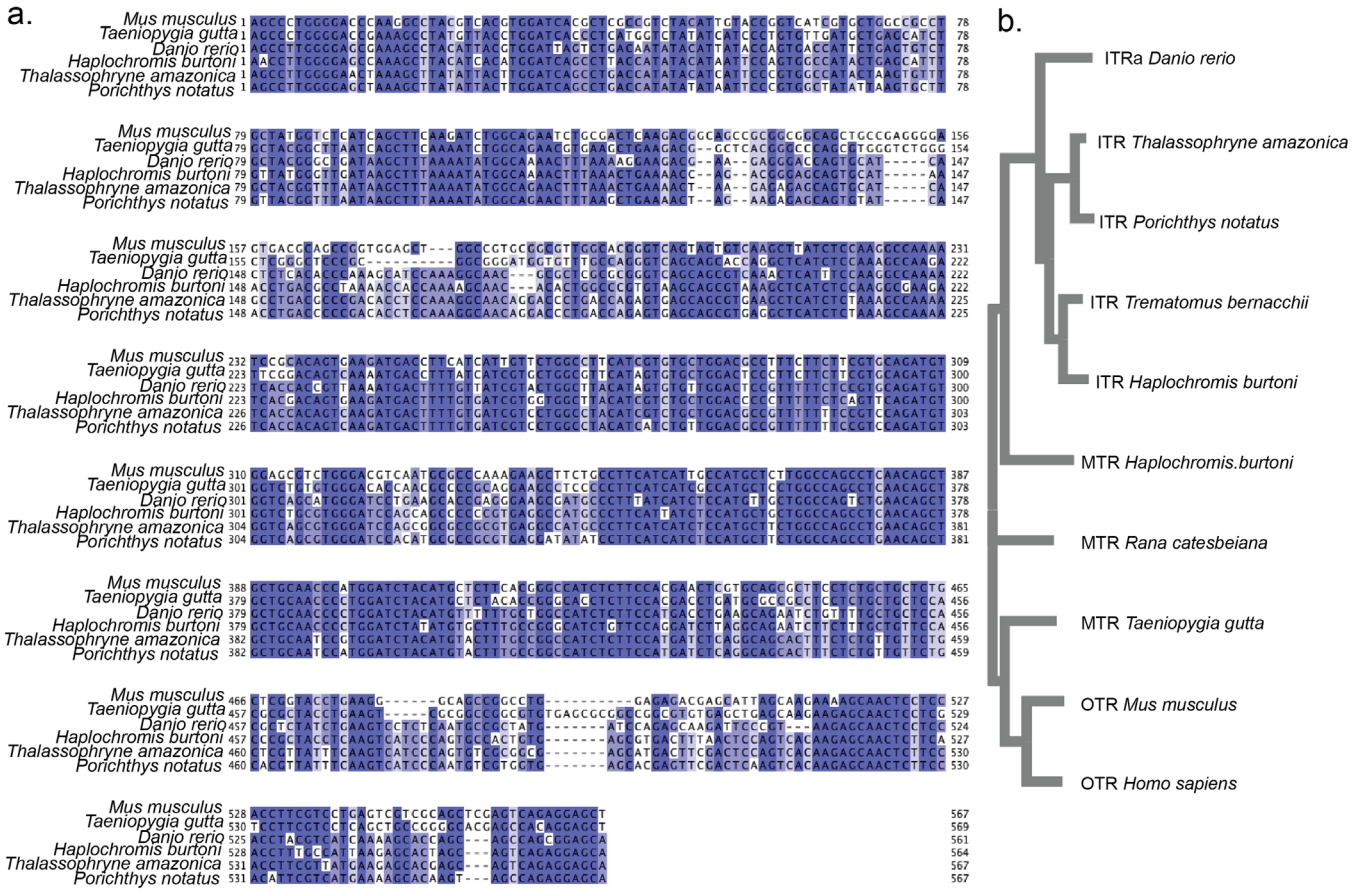

FIGURE 2 Sequence alignment and phylogenetic comparison of isotocin receptor (ITR) in plainfin midshipman (*Porichthys notatus*) with ITR in other teleost fishes, mesotocin receptor (MTR) found in amphibians, reptiles, and birds, and oxytocin receptor (OTR) found in mammals. (a) Multiple sequence alignment of the ITR region amplified in midshipman to other vertebrates. (b) Phylogenetic tree of oxytocin-like receptor sequences across vertebrates that was generated using maximum-likelihood based on alignments via ClustalW

(Figures 3b,c, 4c and 5a,d). Expression was moderate or light in Dl anterior and posterior to the anterior commissure, respectively, and in the central zone (Dc) (Figures 3b–e, 4c and 5a,d,g). Label in the posterior zone (Dp) was only light (Figures 3d and 5g).

Receptor expression was also present throughout the subpallium, area ventralis of the telencephalon (V), in ventral (Vv), dorsal (Vd), supracomissural (Vs), intermediate (Vi) and postcommissural (Vp) nuclei. Staining was very light throughout Vv (Figures 3b and 4c), and varied from moderate to light in Vs, Vp, and Vi (Figures 3c,d and 5a,d,g). Variable label in Vd allowed the recognition of dorsal and ventral divisions (Vdd, Vdv). While label was light in Vdv, Vdd was among the few brain regions with dense ITR label (Figures 3b, 4c and 5a).

Consistent with previous transcriptomic analyses in midshipman (Tripp et al., 2018), we identified dense ITR expression in both anterior (PPa) and posterior (PPp) regions of the parvocellular POA (Figures 3c–e and 5a,b,d,e,g,h). However, within the magnocellular region of the POA (PM), we only observed light ITR expression in both the magnocellular (PMm) and gigantocellular (PMg) populations (Figures 3e and 5d,e,g,h).

Finally, we identified light labeling of ITR in the suprachiasmatic nucleus (SCN, Figures 3e and 5g; also see Feng et al., 2019 for cytoarchitecture) that receives visual input from the retina in midshipman and other teleosts (Foran et al., 1997) and is presumed to regulate rhythmic behaviors, including vocal courtship in midshipman (Feng & Bass, 2016).

In general, ITR sites overlapped locations of putative IT fibers and were comparable in density, as detailed in Goodson et al. (2003; Figure 3a–e). For example, in areas of the subpallium where there was abundant receptor expression such as Vdd, we also found dense fiber staining and in areas of minimal transcript expression in Dm and Dl, we also found sparse fiber labeling. Consistent with Goodson et al. (2003), putative IT somata in the entire brain were only located in the PPa, PPp and PM regions of the POA (solid black circles, Figures 3c–e and 5c,f,i).

3.3 | ITR and IT in diencephalon

Rostrally, the ventral tuberal nucleus (vT) of the anterior hypothalamus, which innervates the PAG and is a vocally active site (Goodson & Bass, 2000a; Kittelberger et al., 2006), exhibited moderate ITR staining (Figures 3e and 5g). The central posterior nucleus in the dorsal thalamus (CP, Figures 3f and 6a) and the anterior tuberal nucleus in the

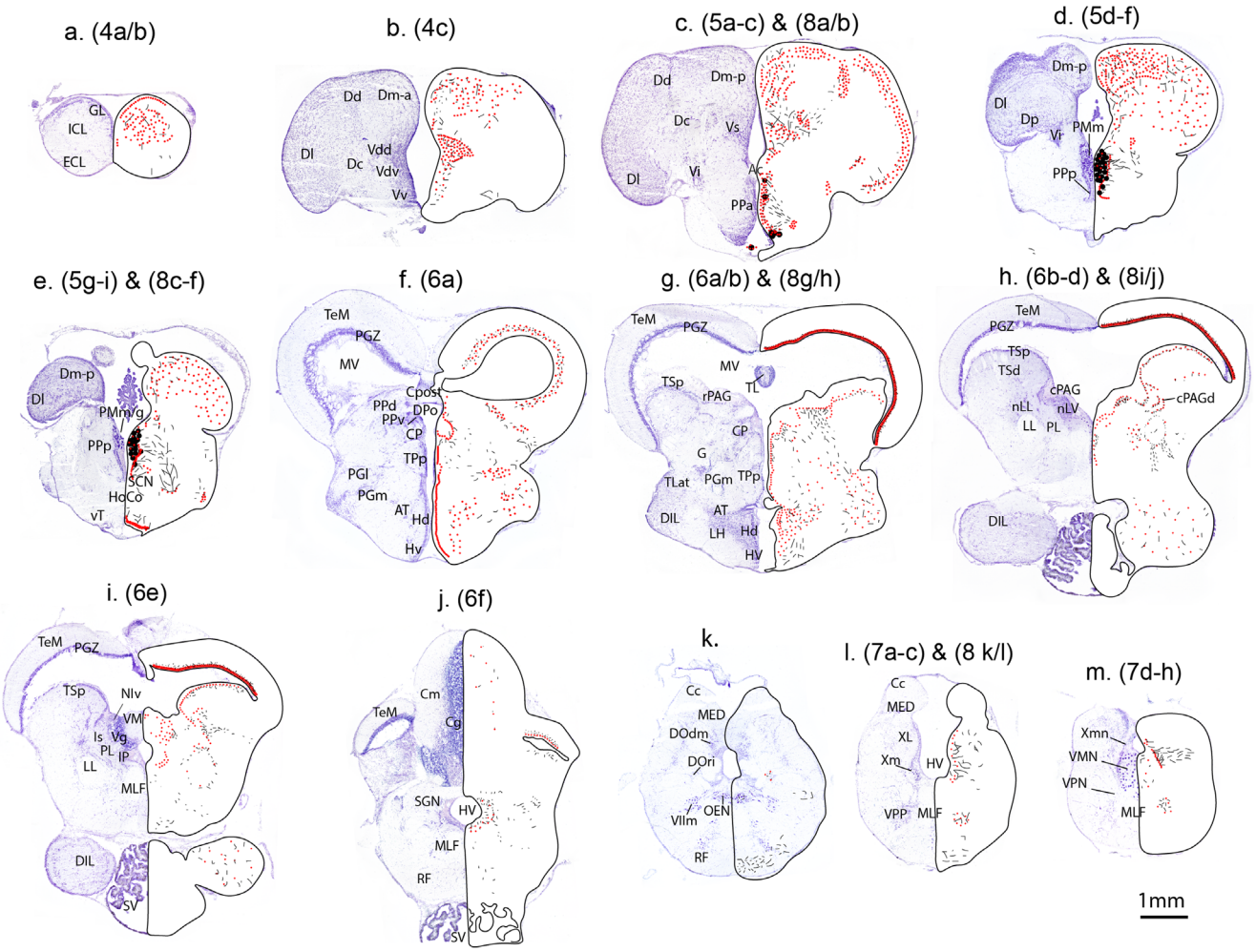

FIGURE 3 Distribution of isotocin receptor (ITR) and putative IT (oxytocin-like) label in the brain of a type I male plainfin midshipman. Nissl-stained, transverse coronal sections (left) and corresponding line drawings (right) illustrate the distribution of ITR mRNA (red stippling) and putative IT immunoreactive somata (solid black circles) and fibers (black lines) throughout midshipman brain at the approximate levels shown in Figures 4–7. Labels above sections indicate figure number and panel corresponding to each level shown. Levels shown are olfactory bulb and rostral telencephalon (a and b); caudal telencephalon and preoptic area (c–e, rostral diencephalon and midbrain (f and g); caudal diencephalon and midbrain (h and i); and far caudal midbrain and hindbrain (j–m). Scale bar is 1 mm. See list of abbreviations for more information

hypothalamus (AT, Figures 3f,g and 6a) that are upstream targets of auditory midbrain efferents (Bass et al., 2000) exhibited moderate ITR (AT is also a vocally active site; Goodson & Bass, 2000c). Moderate label also occurred in the dorsal posterior nucleus (DPo; Figures 3f and 6a). Dense ITR staining occurred in the periventricular nucleus of the posterior tuberculum (TPp, Figures 3f,g and 6a). As with CP and AT, TPp is an integral nucleus within the central auditory network of midshipman, in this case providing dopaminergic input to CP as well as the auditory epithelium of the inner ear and the octavolateralis efferent nucleus, a rostral hindbrain nucleus that also innervates the auditory periphery (Forlano et al., 2014; Perelmuter & Forlano, 2017). Moderate receptor staining was also present in a ventral division of the habenula (Hav, Figure 6a). Label was dense in the dorsal (Hd) and ventral (Hv) zones of the periventricular hypothalamus (Figures 3f,g and 6a), but light in the lateral hypothalamus (LH; Figure 3g), medial and lateral divisions of nucleus preglomerulosus (PGm, PGl; Figures 3f,g and 6a, torus lateralis (TLa, Figure 3g) and the diffuse nucleus of the inferior lobe (DIL, Figure 3h,i). Putative IT fiber label was moderate to light in the above diencephalic regions.

3.4 | ITR and IT in midbrain

Isotocin receptor labeling was concentrated in several midbrain regions directly implicated in the control of vocal behavior. The PAG, which gates descending input from sites in the vocal forebrain to hindbrain pattern generating neurons (Kittelberger et al., 2006), expressed moderate ITR (Figures 3g,h and 6b,c). Label was generally light adjacent to the lateral lemniscus (LL, Figure 3h,i) that carries ascending hindbrain auditory and lateral line efferents to the torus semicircularis (TS; Bass et al., 2000; Weeg & Bass, 2002). The nucleus of the lateral lemniscus, which receives direct input from the auditory division of the TS (Bass et al., 2000), also showed moderate ITR expression (nLL, Figure 3h), while light to moderate label was in the nearby

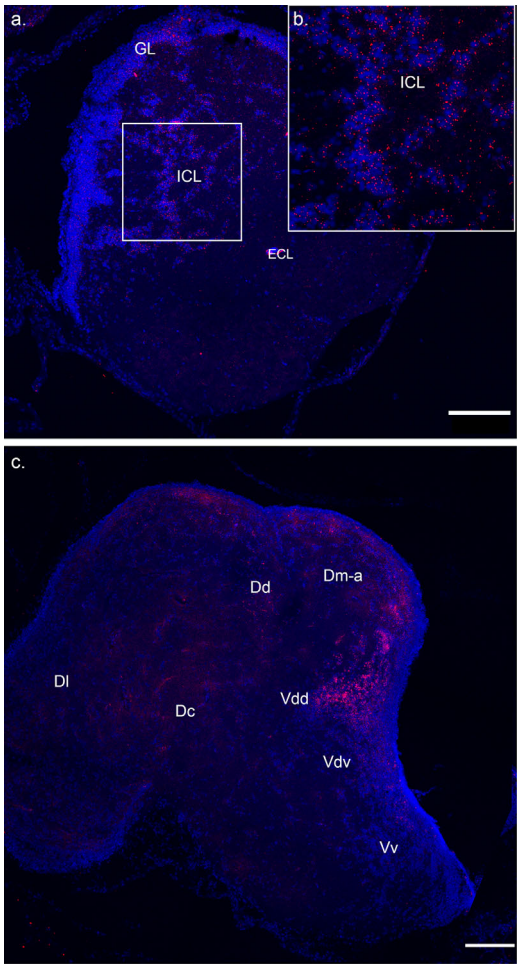

FIGURE 4 Isotocin receptor (ITR) mRNA in the olfactory bulb and rostral telencephalon of a type I male plainfin midshipman fish. Representative ITR mRNA (red) in situ hybridization staining with DAPI nuclear stain (blue or artificially colored purple for contrast enhancement) providing cytoarchitectural detail. (a) ITR staining within the olfactory bulb is most pronounced in the internal cell layer (ICL). (b) Higher magnification view of boxed ICL region in (a). (c) Label is dense in the dorsal division of the dorsal nucleus of area ventralis (Vdd), but much lighter in the ventral division (Vdv) and in the ventral nucleus (Vv). Moderate label is present in the dorsal (Dd) and medial (Dm) zones of area dorsalis, but lighter in its lateral (Dl) and central (Dc) zones. Scale bars are 150 μm. See abbreviation list for more information

paralemniscal tegmentum (PL; Figures 3h,i). Not only do past neurophysiological and neuroanatomical studies implicate PL as a major midbrain site within the vocal network of midshipman (Bass et al., 1994; Kittelberger & Bass, 2013), but it also receives other neuropeptidergic inputs (e.g., arginine vasotocin; Goodson & Bass, 2000c). The periventricular layer of the TS (TSp, Figures 3h,i and 6b,e), a narrow band of somata that appears to be a major source of efferents from the auditory division of the TS (Bass et al., 2000), exhibited light receptor expression. The deep layer of the TS (TSd, Figures 3h and 6b,e), a narrow band of somata that appears to be a secondary integration site for auditory and lateral line information (Bass et al., 2000), exhibited light receptor expression. Lastly, moderate ITR label was in the rostral tegmental nucleus, which lies immediately ventral to the caudal PAG (not shown, but see Pengra et al., 2018) and dense ITR label in the periventricular gray zone of the tectum (PGZ, Figures 3f–j and 6b,e) that is strongly implicated in visual processing (Striedter & Northcutt, 1989).

In general, putative IT fibers overlapped all midbrain sites with ITR label (Figure 3f–j; also see Goodson et al., 2003).

3.5 | ITR and IT in isthmal region and hindbrain

Isotocin receptor is widely distributed within the hindbrain. Moderate to light expression is found at isthmal levels, within the isthmal nucleus (Is, Figures 3i and 6e; not to be confused with tectal-recipient nucleus isthmi; for cytoarchitecture, see Bass et al., 1994, 2000) and the region of the central gray that includes the isthmal periventricular nucleus (IP, not shown; for cytoarchitecture, see Bass et al., 1994 and Tripp & Bass, 2020). Both of these nuclei are part of the central vocal network; they receive input from the PAG (Kittelberger & Bass, 2013) and have labeled terminal-like boutons via transneuronal biocytin transport after vocal nerve labeling (Bass et al., 1994).

Clusters of ITR labeled cells were also observed in the granule cell layer of the corpus of the cerebellum and the eminentia granularis (Cg and EG, respectively; Figures 3j and 6f; also see Bass et al., 2000 for cytoarchitecture). Within the octavolateralis column, clusters of ITR label were found within two populations, the inner ear-recipient rostral intermediate division of the descending octaval nucleus (DOri, Figure 3k) and the lateral line-recipient nucleus medialis (MED, Figures 3k,l and 7a) (see Bass et al., 2000; Weeg & Bass, 2002 for cytoarchitecture). The DOri is a likely site for vocal-acoustic integration, as filled somata are observed after vocal and auditory nerve fills, as well as after biotin injections into the TS or PAG (Bass et al., 1994, 2000; Kittelberger & Bass, 2013). Dense ITR expression appeared over somata within the periventricular layer of the vagal lobe (XL; Figures 3l and 7d). Sparse IT fibers, but no ITR, were also observed within the octavolateralis efferent nucleus (OEN; Figure 3k), which receives inputs from the vocal pattern generator, transmitting a corollary discharge signal to the inner ear (Chagnaud & Bass, 2013).

Receptor expression appeared within two nodes of the vocal pattern generator, duration-coding VPP neurons and amplitude-coding VMN motoneurons that innervate the sound-producing muscles (Chagnaud et al., 2011, 2012). As in past analyses, the exact identity of these neurons is best confirmed using a second marker provided by either transneuronal tract-tracing (Bass et al., 1994) or an indicator of neural activity, such as pS6 (Tripp et al., 2020). Using pS6 (see below, Section 3.6), we confirmed ITR in the VPP and VMN (Figures 3l,m). Label was dense in VPP (Figure 7a,b). Although light ITR expression was scattered throughout the VMN (Figure 7d,e), clusters of moderate label appeared over the somata of a far dorsal population of small motoneurons located at the rostral pole of the VMN (Figure 7g,e; see Bass et al., 1994 for cytoarchitecture).

As in other brain regions, putative IT fibers overlapped hindbrain sites with ITR label. Of special note in regard to the vocal pattern

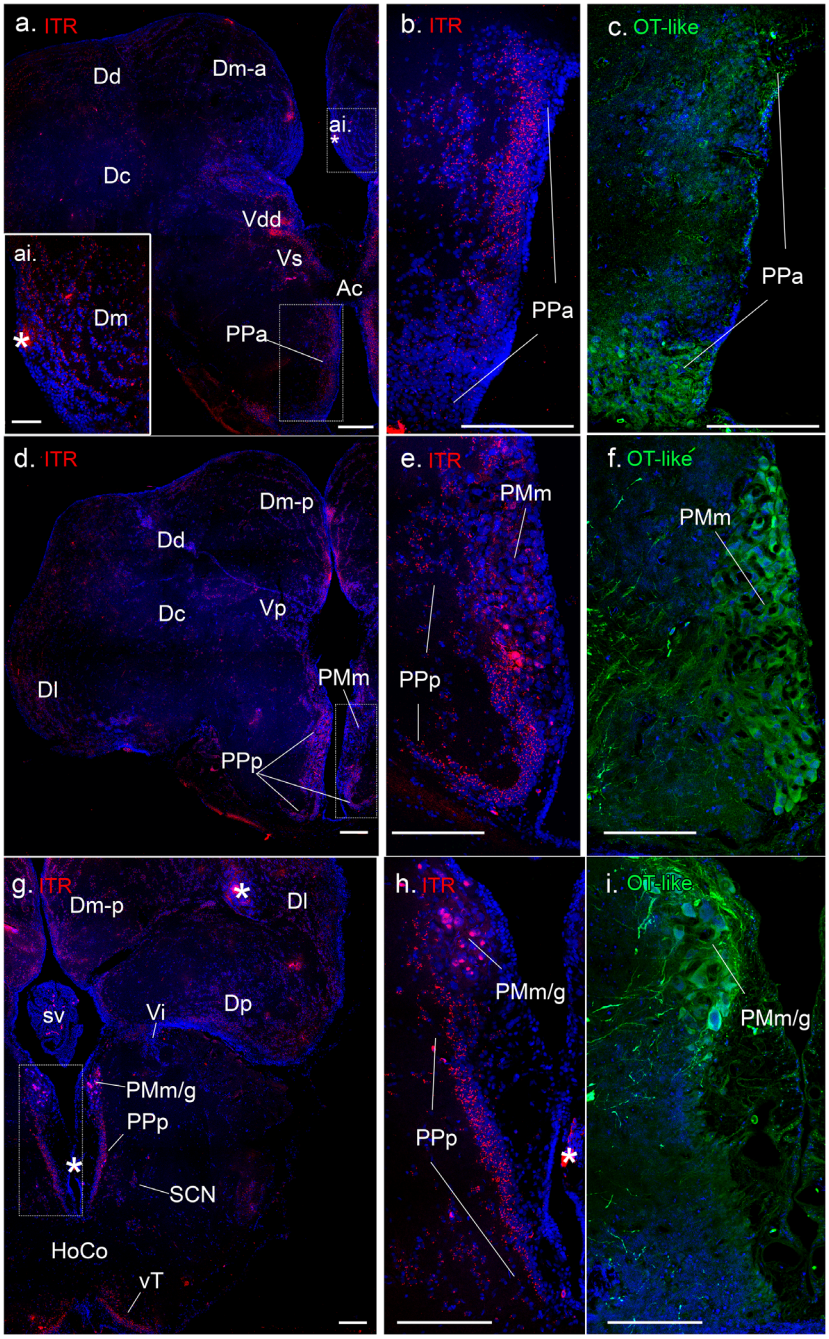

FIGURE 5 Isotocin receptor (ITR) mRNA (red) and putative isotocin (IT)/oxytocin-like (OT-like;green)-containing somata and fibers in the telencephalon and preoptic area (POA) of a type I male plainfin midshipman fish. Representative ITR mRNA in situ hybridization (a, b, d, e, g, h) and putative IT (c, f, i; alternate sections for b, e, h, respectively) staining with DAPI nuclear stain providing cytoarchitectural detail (blue, a–i). Note that e and f highlight PPp and PMm in far-right part of the image. Receptor label is dense throughout much of the anterior parvocellular POA (PPa; a and b), but light in the ventral region where IT somata are present (b and c). Dense ITR label is also present in the ventral region of the posterior parvocellular POA (PPp, d and e), but light in the more dorsal region adjacent to magnocellular IT somata within the magnocellular POA (PMm) (e and f). Farther caudal, ITR label is dense in PPp (g and h) and generally moderate in magnocellular and gigantocellular regions of POA (PMm/g), which includes IT somata (h and i). Scale bars are 150 μm, except in the ai insert (top left panel), where it is 50 μm. See abbreviation list for more information. Asterisk (*) denotes the same location of an artifact in (a) and (ai), (g) and (h). Also artifact in Dl (g)

generator is that putative IT label was found in VPP and VMN. In contrast, there was no convincing evidence of either ITR or IT label in the column of pS6-labeled vocal pacemaker neurons (VPN) that is immediately ventrolateral to VMN (Figures 3m and 7h) and determines motoneuron firing rate (Bass & Baker, 1990; Chagnaud et al., 2011).

3.6 | Role of ITR neurons in vocal courtship

To determine whether ITR mRNA positive (+) neurons within the vocal network were activated during humming, we utilized pS6 as a marker for recently activated neurons as done in prior midshipman studies (Tripp et al., 2020), with a focus on major nodes within the descending vocal control pathway that exhibit ITR expression (see Figure 1b and Section 1). Robust pS6 expression was found throughout the POA in both humming and silent (controls) males (Figure 8a–d), but only the PPa of humming males showed a significant increase in the number of ITR+ neurons that coexpressed pS6 (Figure 8m; PPa, ($t(8) = 4.56$, $p < .01$; PPp, $t(7) = 0.87$, $p = .57$), PMm/g, $t(8) = 0.36$, $p = .73$). The vT in the anterior hypothalamus (Figure 8e,f), which innervates the PAG (Goodson & Bass, 2002; Kittelberger et al., 2006), showed a similar profile as the PPa (Figure 8m; $t(8) = 2.79$, $p = .038$). In contrast, the nearby anterior hypothalamic AT nucleus (Figure 8g,h) that is both a part of the ascending auditory system and a vocally active site

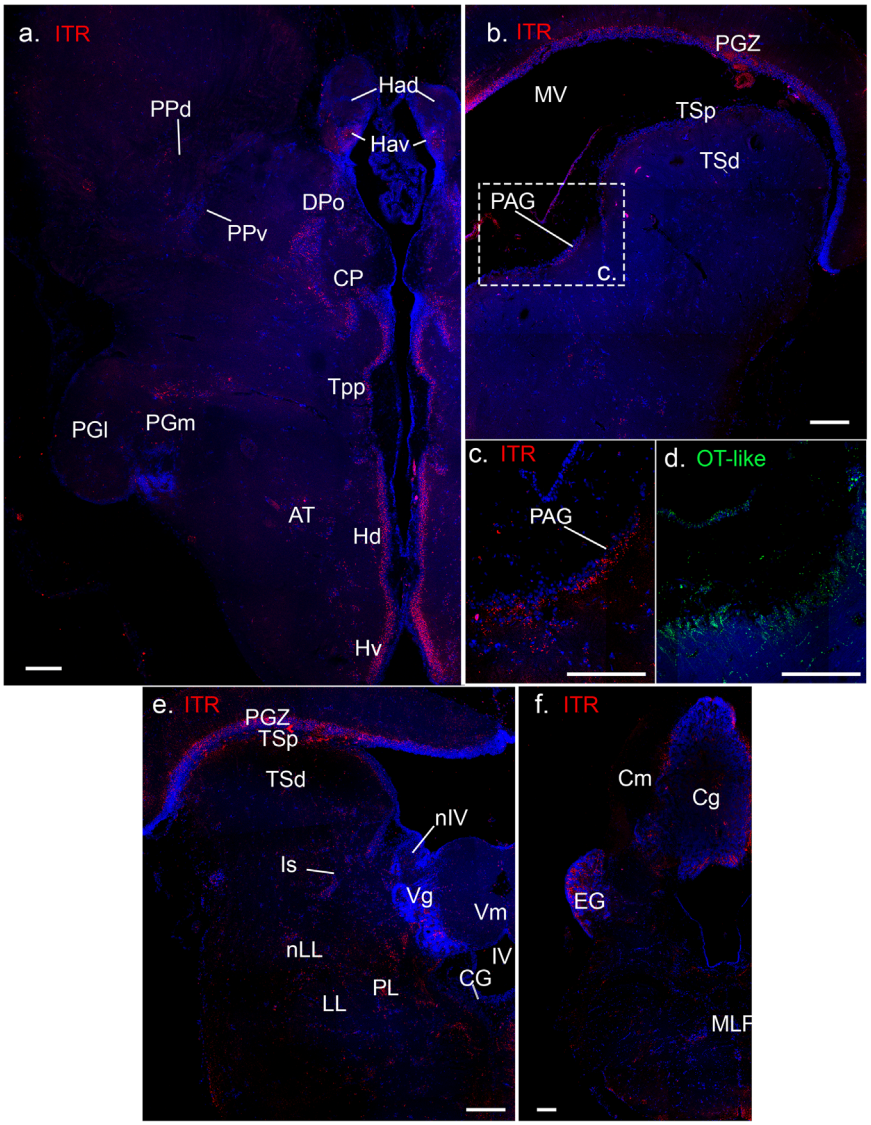

FIGURE 6 Isotocin receptor (ITR) mRNA (red, a–c, e, f) and putative isotocin (IT)/oxytocin-like (OT-like) containing fibers (green, d) with DAPI nuclear stain (blue, a–f) in a type I male plainfin midshipman fish. At rostral levels of the diencephalon (a), ITR label is apparent in the central posterior (CP) and dorsal posterior (DPo) nuclei of the dorsal thalamus, dorsal and ventral zones of the periventricular hypothalamus (Hd, Hv), periventricular nucleus of the posterior tuberculum (TPp), and lateral and medial divisions of nucleus preglomerulosus (PGl, PGm). In the midbrain, ITR label is largely moderate in the periventricular grey zone of the tectum (PGZ; b and e), periventricular layer of the torus semicircularis (TSp; b and e), deep cell layer of the TS (TSd, e), paralemniscal tegmentum (PL, e), and periaqueductal gray (PAG; b, c). Putative IT fibers are also present at PAG levels (d, alternate section to c). In the rostral hindbrain (f), ITR is light to moderate in the eminentia granularis (EG) and granule cell layer of the corpus of the cerebellum (Cg). Scale bars are 150 μm. See abbreviation list for more information

(Bass et al., 2000; Goodson & Bass, 2002) did not exhibit any difference in the number of pS6-ITR neurons between humming and non-humming males (Figure 8m; $t(8) = 0.52$, $p = .72$). Finally, the PAG (Figure 8i,j), which directly activates the hindbrain vocal pattern generator (Kittelberger et al., 2006), exhibited a pattern similar to PPa and vT (Figure 8m; $t(8) = 2.45$, $p = .04$).

Only humming males exhibited pS6 expression in all three compartments of the vocal hindbrain pattern generator–VPP, VPN, and VMN. Within premotor populations, only VPP neurons, which coexpress ITR and pS6 (Figure 8k), showed a significant increase in the number of ITR+ neurons in humming males (Figure 8k–m; $t(8) = 21.91$, $p < .001$). Although ITR and IT fibers were expressed in the vicinity of the VPN (Figure 7h), pS6 revealed that ITR was not coexpressed with this neural activity marker in humming animals. Receptor-expressing cells were found near the VPN column, but these were not labeled with pS6 during humming. Coexpression of pS6 and ITR in humming males was also concentrated over somata within the VMN's rostral, far dorsal population of small motoneurons, although it was also evident over the remaining, more extensive population of motoneurons that are several fold larger (Figure 7e). Intriguingly, we also consistently found pS6 expression in smaller ITR neurons directly adjacent to the VMN in locations overlapping the distribution of GABAergic cells that provide dense inhibitory input to the VMN (Chagnaud et al., 2012).

4 | DISCUSSION

Neuropeptide signaling, including action by IT and its homologue OT, is a potent modulator of vocal behavior across species (Donaldson & Young, 2008; Goodson, 2011; Goodson & Bass, 2001; Insel & Young, 2000). In the current study, we evaluated whether sites of putative IT action in the brain are associated with vocal courtship behavior in midshipman fish. First, we demonstrate that ITR mRNA is widely distributed in vocal motor, auditory, and neuroendocrine nuclei. This includes forebrain (PPa, vT), midbrain (PAG) and hindbrain (VPP) neurons and VMN motoneurons that together form a descending pathway activating output from the vocal hindbrain pattern generator to vocal musculature (Chagnaud et al., 2011; Goodson & Bass, 2000a;

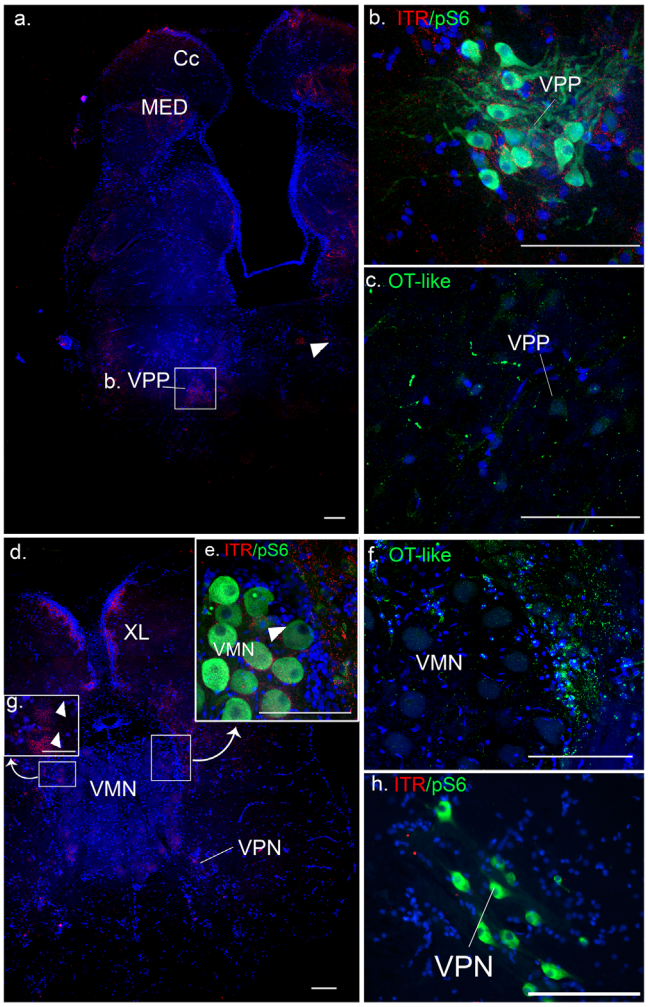

FIGURE 7 Isotocin receptor (ITR) mRNA (red; a, b, d, e, g, h), putative isotocin(IT)/oxytocin-like (OT-like) fibers (green; c and f), ITR with pS6 immunoreactivity, a neural activity marker (green; b, e, h), and DAPI nuclear stain (blue) in hindbrain of humming, type I male plainfin midshipman fish. Boxes in (a) and (d) indicate higher magnification views of enclosed regions in (b) and (e, g, h), respectively. Isotocin receptor mRNA and putative IT fibers (c and f; alternate sections to b and e, respectively) are moderately expressed within vocal prepacemaker nucleus (b and c; VPP; confirmed by pS6 in (b)). However, ITR mRNA is sparce in the vocal motor nucleus (d–g, VMN), except for a population of small motoneurons (arrowheads; b, e, g). ITR mRNA is undetectable in vocal pacemaker neurons (h, VPN). All scale bars are 150 μm, except for (g) where the scale bar is 75 μm

Kittelberger et al., 2006). This suggests that IT might directly modulate motoneurons that drive vocal production as in tetrapods (Leung et al., 2011). Of note is the absence of ITR from the VPN node of the pattern generator that sets the firing rate of VMN motoneurons and, in turn, the contraction rate of vocal muscles that determines the pulse repetition rate/fundamental frequency (PRR) of calls (Chagnaud et al., 2011). Consistent with this finding, the PRR of fictive calls (vocal nerve recordings of evoked potentials that reflect the synchronous firing of VMN motoneurons and mimic the temporal properties of natural calls) is insensitive to the influences of neuropeptides and steroids (Goodson & Bass, 2000b; Remage-Healey & Bass, 2004, 2007), suggesting a general insensitivity of this vocal character to steroid and peptide hormone influences. In the second experiment, we show that multiple populations of ITR neurons within the descending vocal control pathway are active during humming–PPa, vT, PAG, VPP, and VMN. Thus, we propose that IT modulates humming behavior in several ways. First, it acts on the neuroendocrine-rich POA, which is connected to other forebrain sites in the vocal and auditory networks to put animals in a more permissive physiological state during courtship for humming to occur (Goodson & Bass, 2002). Second, IT action in both the anterior hypothalamus (vT) and its downstream target, the midbrain PAG, likely has a directly influence on the activation and temporal patterning of vocalization via the PAG's connections to the ITR-containing vocal hindbrain that codes for call duration and amplitude (VPP and VMN) (Goodson & Bass, 2002; Kittelberger & Bass, 2013; also see Kittelberger et al., 2006 and Chagnaud et al., 2011, 2012 for neural coding patterns in these sites).

4.1 | IT signaling and vocal courtship behavior

In general, our findings reveal that IT-signaling likely occurs throughout a vocal network that is conserved across vocal tetrapods (Figure 1b and Section 1). Below, we consider each of the major functional compartments within this network that would contribute to the behavioral state of vocal courtship.

4.1.1 | Forebrain

Consistent with previous studies of teleosts, the midshipman POA is a major site of ITR expression, including subdivisions that are the sole location in the brain of putative IT-synthesizing neurons (Hausmann et al., 1995; Huffman et al., 2012; O'Connell et al., 2012; Tripp et al., 2018; van den Dungen et al., 1982; Venkatesh & Brenner, 1996). While all POA subdivisions exhibit pS6 expression in both humming and nonhumming type I males, only the PPa exhibits greater activity in humming males. The exact function of these neurons is unclear, but tract-tracing and brain stimulation strongly implicate its role in modulating vocal activity in midshipman and a closely related toadfish species (Fine & Perini, 1994; Goodson & Bass, 2002). We cannot, however, rule out that PPa-pS6 label is attributed to a male hearing their own calls via reciprocal connections of PPa with ITR-containing (+) auditory nuclei (CP, AT) that are part of the ascending auditory system (Bass et al., 2000; Goodson & Bass, 2002).

The PPa has strong reciprocal connections with the anterior hypothalamus, vT, another central node in the vocal-acoustic network (Goodson & Bass, 2000a, 2002) and, as shown here, an ITR+ site (see Figure 1b). Especially important is that neurophysiological studies show that this nucleus directly activates the midbrain PAG that, in turn, drives hindbrain output to the sound-producing vocal muscles (Kittelberger et al., 2006). Neurophysiology also demonstrates that direct infusion of an OT antagonist to the vT likely modulates natural

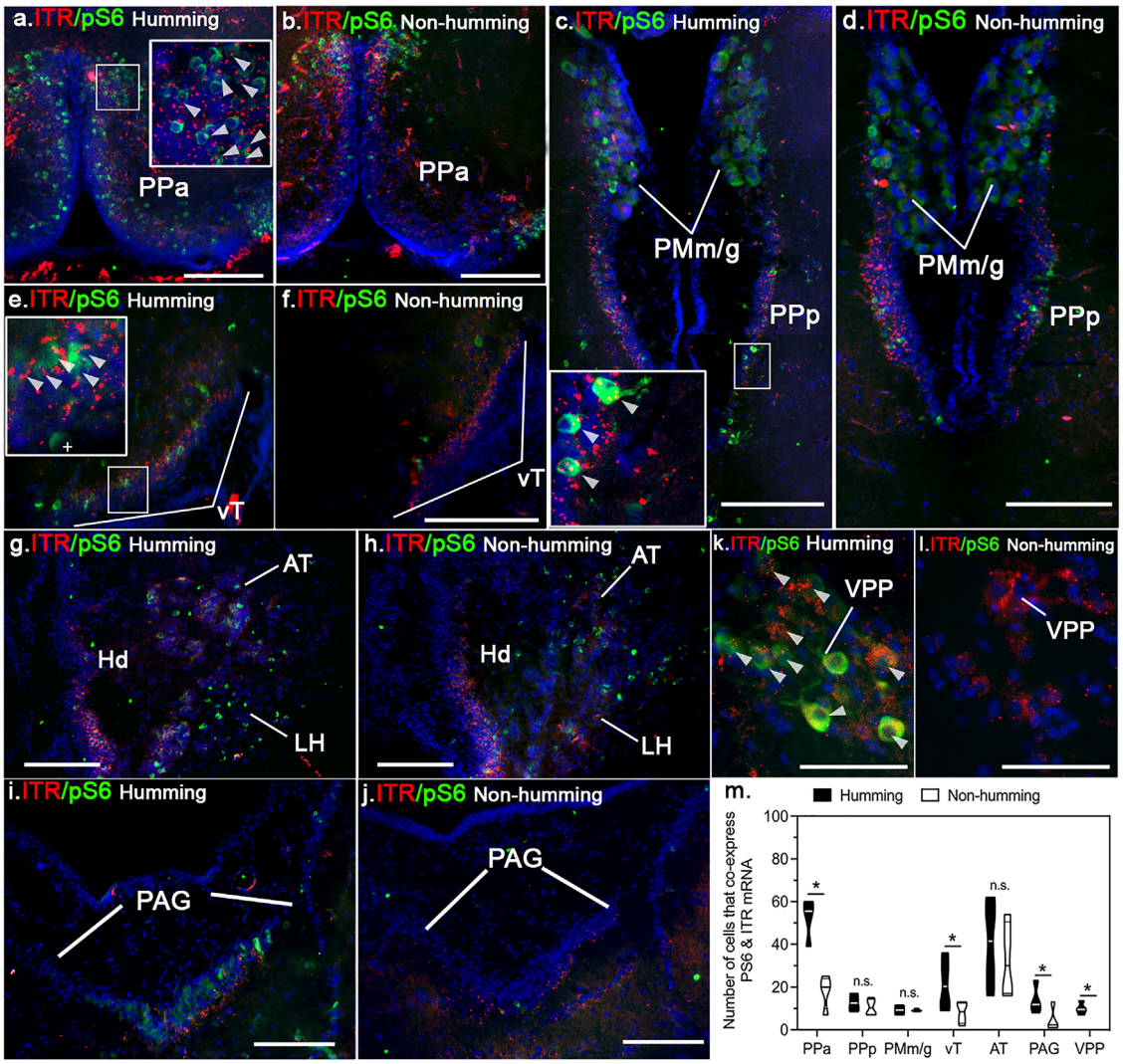

FIGURE 8 Representative differences in neural activity marker (pS6 immunoreactive cells) in isotocin receptor (ITR) mRNA (visualized through in situ hybridization) expressing cells within the vocal motor network of a humming ($n = 4$; a, c, e, g, i, k) or non-humming ($n = 5$; b, d, f, h, j, l) type I male plainfin midshipman. Violin plots (lines represent median and quartiles) illustrate differences in the number of cells that express both ITR mRNA and pS6 is also shown (m). Scale bars are 150 μm, except for VPP images (k and l), where they are 75 μm. Inserts (a, c, e) are high magnification images of regions (white square) within the PPa, PPp, and vT that illustrate coexpression of pS6 and ITR mRNA. Coexpression shown with arrowheads. +: a pS6 positive cell in vT insert (e) that does not express ITR mRNA. See abbreviation list for more information

agonistic grunting behavior in a sex- and male morph-specific manner. Vocal nerve recordings of fictive grunts show that the OT antagonist induces a significant and pronounced increase in the number of grunts evoked in females and type II males following electrical stimulation in the vT, but has only a marginal, nonsignificant effect in type I males (Goodson & Bass, 2000a). Given this weak effect in the humming male morph, it is possible that ITR neurons in this nucleus integrate information about social context to facilitate appropriate changes in behavior. In this sense, IT action in these neurons may put type I males in a more permissive state to produce courtship vocalizations. Consistent with this interpretation, transcriptomic studies demonstrate that type I males that are behaviorally restricted from courting, and therefore display an alternative mating tactic of cuckolding (the only reproductive tactic known for type II males), exhibit significantly lower ITR levels in the POA and contiguous AH that includes vT (Tripp et al., 2018). More broadly, the idea that the vT nucleus integrates information about social contexts is consistent with work across teleosts. When subordinate male African cichlids (*Astatotilapia burtoni*) are put in a behavioral context that permits social ascension, there is an increase in immediate early gene (IEG) in the vT (Maruska et al., 2013).

As mentioned above for PPa, another possibility is that ITR neurons are activated in response to hearing one's own vocalizations. The IEG cFos shows that the vT and the dorsal thalamic auditory nucleus, CP, which are reciprocally connected (Goodson & Bass, 2002), are weakly and strongly activated, respectively, in type I males exposed to underwater playbacks of hums (other call types were not tested) (Petersen et al., 2013). We do not find greater neuronal activation in

another anterior hypothalamic auditory-recipient nucleus, AT, when males are producing courtship hums. Interestingly, the hum playback studies show a pronounced increase of cFos expression in AT. Interpretation of these results in the context of audition driving some of the results we report may be confounded, in part, by the design of the playback experiments compared to the ones conducted here. Type I males in the playbacks were held in a mesh enclosure in the middle of a playback arena, whereas all males in the current study were housed under conditions conducive to active nest defense as well as successful female courtship and mating (Brantley & Bass, 1994; Tripp et al., 2018, 2020). In addition, the hum playbacks lasted for 30 min, while the males in our study hummed for < 15 min. Accordingly, differences in neural activity in AT, like the PPp that also shows no significant increase in activation of ITR+ neurons, may only be found after a protracted period of singing. At the same time, the AT in teleosts may just contribute to modulating the behavioral state of animals. This is consistent with work in mammals showing that the VMH, which has been compared to AT (Forlano & Bass, 2011, also see Table 3), is part of circuitry that modulates aggression and mating behavior (Flanigan & Russo, 2019; La Vaque & Rodgers, 1975). Indeed, findings from European starlings suggest that IEG activity in the VMH is constitutively high when they are defending nesting sites during the breeding season (Heimovics & Riters, 2006). In our study, all males were collected during the breeding season from nest sites and continued to occupy nests under our housing conditions, a behavioral state that is consistently linked to aggressive defense of nests in addition to the vocal courtship behavior studied here (Brantley & Bass, 1994; Lee & Bass, 2006; Tripp & Bass, 2020).

Other ITR+ regions within the POA remain constitutively active at nighttime in type I males. That is, there are no differences in patterns of pS6 expression in the PPp and PMm/g nuclei between control and humming animals (Figure 8). Interestingly, these PMm/g neurons, and to a lesser extent PPp neurons, contain the primary IT-producing cells in the brain (Figure 5; Goodson et al., 2003). Given that this region contains many of the POA-IT synthesizing neurons, it is also possible that these cells remain "active" during nighttime, regardless of whether an animal is courting or not. One possibility is that constitutive activity modulates suites of behaviors, including vocal communication, that are necessary for reproductive success. In midshipman, ITR expression within the POA and AH dramatically changes with respect to behavioral and reproductive tactic (e.g., courting vs. cuckolding; Tripp et al., 2018). Since levels of this receptor are significantly upregulated in courting males, these active ITR neurons may gate the expression of other behaviors that are specific to nest-holding type I males during the breeding season (see Brantley & Bass, 1994; Tripp et al., 2020). In this sense, other ITR expressing neurons within the POA might alter the physiological state of the animal, and therefore place the animal in a more permissive state for humming to occur. This is consistent with reports in other vertebrates that suggest IT action in the POA promotes sexual and reproductive behaviors (Black et al., 2004; DeAngelis et al., 2018; Kleszczyńska et al., 2012; O'Connell et al., 2012).

Although our study focused on pS6 (e.g., neural activity marker) expression in ITR expressing neurons, we expect that activation of IT neurons within the POA likely plays an important role in modulating courtship, including vocalization. Indeed, IT in the POA is associated with reproductive-related behaviors across teleost fishes (see above). In our study, we identified a robust increase in the number of pS6+ cells within the PPa, a site of IT synthesizing neurons (Figure 5c), when animals were humming (Figure 8a,b,m). Given that all of these studies suggest activation of neurons that synthesize OT-like peptides can influence reproductive behavior (Garrison et al., 2012; Goodson, 2013; Insel, 1997), future investigations should aim to specify the functional role of POA-IT neurons in behavioral actions.

4.1.2 | Midbrain and hindbrain

Reports across vertebrate lineages demonstrate that the PAG plays an important role in the descending control of vocal output (Chen et al., 2021; Kittelberger & Bass, 2013; Tschida et al., 2019). In midshipman, this nucleus not only receives input from vocal forebrain and hindbrain (IP) neurons, but also projects to VPP (Kittelberger & Bass, 2013). Neurophysiology further suggests that the PAG has sparse input into the VMN (Chagnaud et al., 2012; Kittelberger & Bass, 2013). Thus, as in other vertebrates (Tschida et al., 2019), this region has the ability to directly modulate motoneurons as well as premotor regions that define the acoustic structure of calls. Isotocin signaling may be able to modulate vocal output in midshipman via ITR+ neurons in the PAG as well as in its downstream hindbrain targets, all of which are activated during sustained humming activity (see Figure 8; VPP, VMN). How exactly ITR+ neurons in the PAG might modulate vocal courtship in midshipman remains an open question for future study.

Our results also highlight possible IT action in two neuronal populations of the vocal hindbrain, VPP and VMN, both of which are activated during humming behavior. Although we find putative IT fibers and ITR+ cells next to the VPN, these cells do not appear to express pS6 in humming males (Figure 7d,h). Premotor VPP neurons receive inputs from the midbrain PAG and encode vocal duration (Chagnaud et al., 2011; Goodson & Bass, 2002; Kittelberger & Bass, 2013). As mentioned earlier, VPP is also the source of a corollary discharge that informs the auditory periphery about call duration (Chagnaud & Bass, 2013). In this way, IT could modulate auditory encoding directly via action in ITR+ auditory nuclei (DOri, TS, CP, AT) and indirectly via VPP.

We also found ITR and IT fibers within a topographically distinct population of small motoneurons found at the rostral end of the VMN that is mainly comprised of motoneurons that are about three times larger (Bass et al., 1994). While neurophysiology shows that large motoneuron activity direct influences call amplitude (Chagnaud et al., 2012), we have been unable to identify neurophysiological properties for the small motoneurons that might have a distinctive impact on call structure (A. Bass, unpublished observations) that could be affected, in turn, by IT action.

Expression of pS6 was also found in small ITR+ neurons surrounding the VMN that overlap the location of GABAergic neurons previously shown to densely innervate the VMN (Chagnaud et al., 2012; Forlano et al., 2014). Isotocin action here likely influences call duration given

TABLE 3 Expression patterns of oxytocin-like receptor across diverse lineages of vocal vertebrates

Midshipman	Singing mouse[1]	Oscine songbird[2]	Frog[3]
Telencephalon			
Supracommissural, intermediate and posterior nuclei of area ventralis (Vs, Vi, Vp) +	Extended amygdala/bed nucleus of the stria terminalis ?/+	Extended amygdala/bed nucleus of the stria terminalis +	Extended amygdala/bed nucleus of the stria terminalis ?/–
Dorsal and central nuclei nuclei of area ventralis (Vd, Vc) +	Striatum ?	Striatum + (absent in area X)	Striatum +
Ventral nucleus of area ventralis (Vv) +	Septum +	Septum +	Septum +
Preoptic area (POA)			
Anterior parvocellular nucleus (PPa) +	POA (parvocellular nuclei) +	?	Anterior POA +
Posterior parvocellular nucleus (PPp) +			
Magnocellular division of magnocellular nucleus (PM) +	Paraventricular nucleus –		Magnocellular division of PM +
Gigantocellular division of PM (PMg) +			
Diencephalon			
Central posterior nucleus (CP) +	Medial geniculate –	Nucleus ovoidalis +	Central and posterior thalamic nuclei ? Ventromedial thalamic nucleus +
Habenula +	Habenula ?	Habenula +/–	Habenula –
Posterior tuberculum (TPp, TP) +	Substantia nigra/ventral tegmental area or A11[1] ?	Substantia nigra/ventral tegmental area[1] +	Ventral tegmental nucleus +
Anterior tuberal nucleus (AT) +	ventromedial hypothalamus (VMH) +	VMH +/–	VMH +
Midbrain			
Tectum mesencephali (TeM) +	Superior colliculus ?	Optic tectum +	Optic tectum +
Torus semicircularis (TS) +	Inferior colliculus ?	MLd (nucleus mesencephalicus lateralis, pars dorsalis ?	Torus semicircularis +
Periaqueductal gray (PAG) +	Periaqueductal gray ?	Nucleus intercollicularis +/–	Comparable structure unknown
Hindbrain			
Purkinje cells of cerebellum +	Purkinje cells of cerebellum ?	Purkinje cells of cerebellum +	Purkinje cells of cerebellum +
Reticular formation +	Reticular formation ?	Reticular formation ?	Reticular formation ?

(Continues)

TABLE 3 (Continued)

Midshipman	Singing mouse[1]	Oscine songbird[2]	Frog[3]
Rostral intermediate division of descending octaval nucleus (DOri) +	Cochlear nucleus ?	Nucleus angularis ? Nucleus magnocellularis +/−	Dorsal medullary nucleus − Ventral nucleus +
Vocal motor neurons (VMN) +	?	Tracheosyringeal portion of the hypoglossal nucleus (nXIIts) +	Nucleus of the glossopharyngeal (IX) and vagus (X) nerve −

[1]Information compiled from oxytocin receptor expression visualized through receptor autoradiography in singing mice (*Scotinomys teguina and Scotinomys xerampelinus*; Campbell et al., 2009).
[2]Information compiled from mesotocin receptor expression in zebra finch and white throated sparrow visualized through in situ hybridization. Instances of a +/− indicate that expression was found in one songbird but not the other species (Leung et al., 2011).
[3]Information compiled from mesotocin receptor expression in *Rana catesbeiana* and *Rana esculenta* (Acharjee et al., 2004) visualized through in situ hybridization. Nomenclature for auditory nuclei based on Mccormick, 1999 and Wilczynski and Endepols, 2007.
[4]Comparisons of posterior tuberculum to the substantia nigra/ventral tegmental area or all population of diencephalic dopaminergic neurons remain unresolved (Pengra et al., 2018).
Question mark (?) indicates that it is unclear if this has been investigated.
+ Indicates presence of oxytocin-like receptors.
− Indicates absence of oxytocin-like receptors.

GABA's direct modulation of overall activity levels in VMN and, in turn, duration of the vocal motor volley that sets final call duration (Chagnaud et al., 2012). The VMN also receives catecholaminergic and serotonergic inputs (Forlano et al., 2014; Timothy & Forlano, 2020); their influence on VMN physiology remains to be investigated.

Together, these findings highlight three potential ways by which IT could modulate vocal structure: direct action on ITR-expressing neurons in VPP and VMN, and on neuromodulatory (e.g., GABAergic) neurons having direct input to the VMN (Chagnaud et al., 2012).

4.2 | Comparisons with other teleosts

Although several studies report expression levels for ITR in teleost brain (e.g., DeAngelis et al., 2018; Tripp et al., 2018; Weitekamp et al., 2017), there are no brain atlases of ITR expression sites in teleosts comparable in scope to the current report other than an earlier one for an African cichlid, *Astatotilapia burtoni* (Huffman et al., 2012). Like several other cichlid species (Bertucci et al., 2012; Myrberg et al., 1965), *A. burtoni* is also sonic (Maruska et al., 2012). In general, the brain-wide pattern of ITR label reported here for midshipman compares closely to that described for the forebrain and midbrain of *A. burtoni* (Huffman et al., 2012). Specifically, with regards to comparisons of the midshipman's forebrain-midbrain/isthmal vocal-acoustic system, *A. burtoni* exhibits ITR expression in all sites summarized in Figure 1b except for the nLL, Is, and IP. Other than the cerebellum's granule cell layer, the cichlid study did not report any other ITR label in the hindbrain (Huffman et al., 2012).

Shared patterns of ITR label in the POA, vT and PAG of midshipman and a cichlid strongly suggest that nonapeptide modulation and general organization of the vocal system in cichlids will compare closely to that of midshipman where prior neurophysiological studies and the current one, using pS6 as a marker of neuronal activation during vocal behavior, support the role of these regions in vocal mechanisms (Section 4.1).

4.3 | Comparisons with vocal tetrapods

Modulation of vocal behavior by members of the OT family of nonapeptides in the central nervous system is largely conserved across diverse vertebrate lineages (Donaldson & Young, 2008). In addition to work in midshipman (Goodson & Bass, 2000a), blocking mesotocin action decreases the amount of time male songbirds produce courtship songs (Pedersen & Tomaszycki, 2012; but see Goodson et al., 2004; Klatt & Goodson, 2013). Yet, knocking out OT receptors produces more mixed results in mice; some studies report a decrease in ultra-sonic vocalization (Winslow et al., 2000), while others suggest little effects (Mogi et al., 2014). Although the reason for these mixed findings across taxa is unclear, it may be partially due to how OT-like signaling was manipulated (e.g., location and concentration of injection or type of knock-down; Theofanopoulou et al., 2017). Such differences might be attributed to how antagonists were delivered or the specificity and timing of OT knockouts. Furthermore, OT-like peptides may only modulate vocalizations in some contexts, but not others (e.g., directed vs. undirected singing; Pedersen & Tomaszycki, 2012). Regardless, surveying the anatomical expression patterns of OT-like receptors can reveal how this peptide might influence vocal behavior across species.

Anatomical studies across vocal vertebrates, including the current one, highlight where OT-like peptides can act within a conserved vocal network (Table 3). Our findings expand upon evidence showing that OT-like signaling systems in the POA and AH conform to an evolutionarily conserved pattern (Goodson & Kingsbury, 2013; O'Connell & Hofmann, 2012). This is most evident for the paraventricular nucleus and its homologues, including PM/PMg in teleosts (see Forlano & Bass, 2011), which is often considered the primary biosynthetic site for

OT-like peptides in the brain (Mohr et al., 1988). Similar to previous reports for midshipman (Goodson et al., 2003), we confirm that most, if not all, putative IT+ neurons are in the POA. Although these neurons in mammals release OT into the peripheral circulation, they also project to the amygdala and bed nucleus of the stria terminalis to modulate social behaviors in rodents (Landgraf & Neumann, 2004; Ross et al., 2009). We find a similar pattern of putative IT fibers innervating comparable regions in midshipman brain (Table 3, also see Goodson et al., 2003). More importantly, we demonstrate dense ITR expression in many areas where putative IT fibers are present. Together, this suggests a conserved role of the POA-AH as the primary central site for synthesis of IT/OT that can influence social behavior via actions in a large suite of brain regions (Table 3).

Of particular interest to midshipman studies is the midbrain PAG, a critical node connecting the forebrain to the hindbrain vocal pattern generator (see Figure 1b). In birds and mammals, such as midshipman, OT-like receptors are present in the PAG (Leung et al., 2011; Newmaster et al., 2020). Not only are these receptors located in the PAG of vocal learning songbirds, but they are also found in the PAG of rodents that produce innate vocalizations (Leung et al., 2011; Newmaster et al., 2020; Schmidt & Martin Wild, 2014). In the vocal vertebrates studied so far, including midshipman, the PAG has descending input to hindbrain premotor-motor circuits that modulate vocal output (Kittelberger & Bass, 2013; Tschida et al., 2019). Similar to midshipman, studies in songbirds also find OT-like fibers near the PAG (Goodson et al., 2003; Leung et al., 2011). Perhaps OT-like action in the PAG influences vocalizations in a similar manner across species.

The identification of OT-like signaling within the vocal hindbrain of tetrapods is limited and has mixed results (Table 3). Consistent with our findings, motoneurons driving syringeal muscle movements in oscine songbirds have mesotocin receptors (Leung et al., 2011). Ranid frogs, however, lack mesotocin receptors in laryngeal motoneurons that drive vocal output (Acharjee et al., 2004).

5 | CONCLUSIONS

The current study explored the potential role of IT signaling in vocal courtship behavior in a teleost fish. We find that potential IT signaling is widespread in neuroendocrine, vocal and auditory nuclei. Notably, ITRs and putative IT cells and fibers are located throughout the POA-AH of reproductively active midshipman that is an integral part of a more extensive vocal-acoustic network (see Figure 1b). As in other vertebrates, we expect that such signaling puts the animal in a more permissive physiological state for advertisement calling (humming) to occur at nighttime during the breeding season. Given that ITR expression in the POA-AH differs between different male reproductive morphs and behavioral tactics (Tripp et al., 2018), we expect that, as with other hormones, there may be seasonal differences in IT and ITR expression in the midshipman brain. We also find ITRs and putative IT fibers in duration-coding (VPP) and amplitude-coding motor neurons that are activated when the animal is humming. This suggests that IT-signaling can directly modulate two of the most salient aspects of courtship hums that strongly affect female responsiveness to underwater playbacks of computer-synthesized sounds–duration and pattern of amplitude modulation (Brantley & Bass, 1994; McIver et al., 2014; McKibben & Bass, 1998, 2001). Given that major nodes in the vocal motor network of midshipman are shared with vocal tetrapods (Table 3), we expect that OT-like signaling might have a conserved role in modulating vocal courtship across vertebrate lineages.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Eric R. Schuppe and Andrew H. Bass designed the study; Eric R. Schuppe and Margaret A. Marchaterre collected the data; Eric R. Schuppe, Jonathan T. Perelmuter, and Andrew H. Bass analyzed the data; Eric R. Schuppe, Melissa D. Zhang, and Andrew H. Bass drafted figures; Eric R. Schuppe, Melissa D. Zhang, Margaret A. Marchaterre, Jonathan T. Perelmuter, and Andrew H. Bass drafted and wrote the manuscript; all authors revised the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

PEER REVIEW

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