

RESEARCH ARTICLE

Galanin immunoreactivity is sexually polymorphic in neuroendocrine and vocal-acoustic systems in a teleost fish

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Abstract

Galanin is a peptide that regulates pituitary hormone release, feeding, and reproductive and parental care behaviors. In teleost fish, increased galanin expression is associated with territorial, reproductively active males. Prior transcriptome studies of the plainfin midshipman (*Porichthys notatus*), a highly vocal teleost fish with two male morphs that follow alternative reproductive tactics, show that galanin is upregulated in the preoptic area-anterior hypothalamus (POA-AH) of nest-holding, courting type I males during spawning compared to cuckolding type II males. Here, we investigate possible differences in galanin immunoreactivity in the brain of both male morphs and females with a focus on vocal-acoustic and neuroendocrine networks. We find that females differ dramatically from both male morphs in the number of galanin-expressing somata and in the distribution of fibers, especially in brainstem vocal-acoustic nuclei and other sensory integration sites that also differ, though less extensively, between the male morphs. Double labeling shows that primarily separate populations of POA-AH neurons express galanin and the nonapeptides arginine-vasotocin or isotocin, homologues of mammalian arginine vasopressin and oxytocin that are broadly implicated in neural mechanisms of vertebrate social behavior including morph-specific actions on vocal neurophysiology in midshipman. Finally, we report a small population of POA-AH neurons that coexpress galanin and the neurotransmitter γ -aminobutyric acid. Together, the results indicate that galanin neurons in midshipman fish likely modulate brain activity at a broad scale, including targeted effects on vocal motor, sensory and neuroendocrine systems; are unique from nonapeptide-expressing populations; and play a role in male-specific behaviors.

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; DI, lateral zone of D; Dm-p, posterior division of Dm; DODm, dorsomedial division of descending octaval nucleus; DOr1/I, rostral intermediate/intermediate division of descending octaval nucleus; DPO, dorsal posterior nucleus of the thalamus; Hb, habenula; Hd, dorsal zone, periventricular hypothalamus; HoCo, horizontal commissure; HV, hindbrain ventricle; Hv, ventral zone, periventricular hypothalamus; IP, isthmal paraventricular nucleus; IS, isthmal auditory-vocal nucleus; LH, lateral hypothalamus; IL, 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; PGI, lateral division of nucleus preglomerulosus; PGM, medial division of nucleus preglomerulosus; 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; 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; VIlm, 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; Xm, vagal motor nucleus.

KEYWORDS

alternative reproductive tactics, auditory, galanin, neuroendocrine, preoptic area, RRID: AB_142018, RRID:AB_2157629, RRID:AB_2534017, RRID:AB_2534018, RRID:AB_2783794, RRID:AB_477652, RRID:AB_90782, vocal

1 | INTRODUCTION

Galanin (Gal) is a 29-amino acid peptide that was first isolated from porcine intestine (Tatemoto, Rökæus, Jörnvall, McDonald, & Mutt, 1983) and since associated with several functions including promoting release of prolactin (Koshiyama et al., 1987), growth hormone (Murakami et al., 1987; Ottlecz, Samson, & McCann, 1986), and luteinizing hormone (López, Merchenthaler, Ching, Wisniewski, & Negro-Vilar, 1991; Sahu, Crowley, Tatemoto, Balasubramaniam, & Kalra, 1987) from the pituitary, as well as involvement in feeding (Kyrkouli, Stanley, & Leibowitz, 1986; Tachibana et al., 2008; Volkoff et al., 2005) and reproductive behaviors (Benelli et al., 1994; Bloch, Butler, & Kohlert, 1996; Bloch, Butler, Kohlert, & Bloch, 1993).

The distribution of Gal expression in the brain has been mapped for several of the major lineages of bony vertebrates, including teleost fish (Amano, Amiya, Hiramatsu, Tomioka, & Oka, 2009; Anglade et al., 1994; Batten, Moons, Cambre, & Vandesande, 1990; Cornbrooks & Parsons, 1991; Holmqvist & Ekstrom, 1991; Jadhao & Pinelli, 2001; Magliulo-Cepriano, Schreiber, & Blum, 1993; Olivereau & Olivereau, 1991; Prasada Rao, Murthy, Cook, & Peter, 1996; Yamamoto, Maler, & Nagy, 1992), amphibians (Lázár et al., 1991; Olivereau & Olivereau, 1992), reptiles (Jiménez, Mancera, Pérez-Figares, & Fernández-Llèbrez, 1994), birds (Azumaya & Tsutsui, 1996; Jozsa & Mess, 1993; Klein, Jurkevich, & Grossman, 2006), and mammals (Gentleman et al., 1989; Kordower & Mufson, 1990; Skofitsch & Jacobowitz, 1985). Shared patterns for the location of Gal-immunoreactive (ir) neurons and fibers include Gal-ir neuron populations in the preoptic area (POA) and hypothalamus, and Gal-ir processes broadly distributed throughout the brain. Among teleosts, the focus of the current study, Gal-ir somata are limited to fewer regions compared to tetrapods, show a large degree of variability in location as do Gal-ir fibers, and include cases of sexual dimorphism. For example, Gal-ir fiber distributions are reduced in female sailfin mollies (*Poecilia latipinna*) and goldfish (*Carrassius auratus*) compared to males, and male goldfish have a greater number of Gal-ir somata in the parvocellular division of the magnocellular nucleus of the POA and the posterior tuberal nucleus of the posterior tuberculum (Cornbrooks & Parsons, 1991; Prasada Rao et al., 1996). Jadhao and Meyer (2000) describe a Gal-ir population of magnocellular preoptic neurons that are present in female red salmon (*Onchorhynchus nerka*), but not males.

Aside from a general role for Gal neurons in feeding and reproductive behaviors (see earlier references), more recent studies in mice that include optogenetic activation show that Gal-expressing neurons of the medial POA promote parental behaviors (Kohl et al., 2018; Wu, Autry, Bergan, Watabe-Uchida, & Dulac, 2014). Transcriptomic analyses have also implicated a role for Gal in the social behaviors

of teleost fish, where increased Gal expression is associated with territorial, reproductively active males (Partridge, MacManes, Knapp, & Neff, 2016; Renn, Aubin-Horth, & Hofmann, 2008; Tripp, Feng, & Bass, 2018). We recently showed in the plainfin midshipman (*Porichthys notatus*), a highly vocal teleost, that expression of the transcript encoding Gal in the POA-anterior hypothalamus (AH) differs between alternative male reproductive morphs that exhibit widely divergent patterns of courtship and spawning behaviors (Tripp et al., 2018). This led to the current study that maps the distribution of Gal-ir somata and fibers in the midshipman brain.

The midshipman's two male reproductive morphs are distinguishable by a number of behavioral, somatic, hormonal, neuroanatomical, and neurophysiological traits (Bass, Horvath, & Brothers, 1996; Brantley & Bass, 1994; Brantley, Wingfield, & Bass, 1993; Cogliati, Balshine, & Neff, 2014; Fitzpatrick et al., 2016; Goodson & Bass, 2000a; Lee & Bass, 2004; McIver, Marchaterre, Rice, & Bass, 2014; Remage-Healey & Bass, 2004, 2007; also see Feng & Bass, 2017 for a recent review). Type I males are the larger morph that exhibit territoriality (nest building and guarding), acoustic courtship of females, and parental care behaviors. They have a vocal repertoire that includes long (minutes-hours) courtship hums, brief (milliseconds) agonistic grunts that may be repeated in a train lasting seconds, and agonistic growls (seconds) that combine hum and grunt attributes. Type II males are the smaller cuckold morph, while females overlap the two male morphs in size. Type II males do not acoustically court females, neither type II males nor females guard nests or provide parental care, and both type II males and females are known only to produce single grunts.

Our recent transcriptomic analyses reveal that transcripts encoding Gal are upregulated in the POA-AH of type I males engaged in the nest-holding, courtship reproductive tactic compared to type II males engaged in cuckoldry (Tripp et al., 2018). Given the teleost POA includes nonapeptide synthesizing neurons that are part of the AH of amniotes, we have referred to this region in midshipman as the POA-AH (see Bass & Forlano, 2008). To better localize the neuronal populations where Gal might act to influence performance of the above behaviors, we sought to determine if and how Gal-ir systems in the midshipman brain differ within and between the sexes. Complementing prior neuroanatomical studies of midshipman (Feng, Marchaterre, & Bass, 2019; Forlano, Deitcher, & Bass, 2005; Forlano, Marchaterre, Deitcher, & Bass, 2010; Goodson & Bass, 2000b; Goodson, Evans, & Bass, 2003) our analysis of Gal neuroanatomy focuses on the well-established vocal-acoustic (Figure 1) and neuroendocrine circuitry by addressing four questions: (1) Where are Gal-ir neurons and their projections distributed in the brain? (2) Does the pattern of Gal expression differ between the two male morphs and females? (3) Do Gal-ir

neurons coexpress the nonapeptides arginine vasotocin (AVT) or isotocin (IT)? (4) Do Gal-ir neurons coexpress the neurotransmitter γ -aminobutyric acid (GABA)?

We use double-label fluorescent immunohistochemistry (IHC) to determine whether Gal-ir neurons in the POA-AH coexpress the nonapeptides AVT or IT, the teleost homologues of arginine vasopressin (AVP) and oxytocin (OT), respectively. We focus on nonapeptides because of their abundant expression in the POA-AH (e.g., see Foran & Bass, 1998; Goodson & Bass, 2000b for midshipman) and well-established roles in vertebrate social behavior (reviewed in: Goodson, 2013; Kelly & Goodson, 2014; Dumais & Veenema, 2016), including among teleosts that show reproductive behavior plasticity in the form of adult sex or role reversal, or alternative reproductive tactics (e.g., Almeida, Gozdowska, Kulczykowska, & Oliveira, 2012; Godwin, Sawby, Warner, Crews, & Grober, 2000; Godwin & Thompson, 2012; Greenwood, Wark, Fernald, & Hofmann, 2008; Huffman, Hinz, Wojcik, Aubin-Horth, & Hofmann, 2014; Semsar, Kandel, & Godwin, 2001). In midshipman, these hormones also have reproductive morph-specific effects on vocal circuit physiology (Goodson & Bass, 2000a). Finally, because the majority of Gal neurons in multiple subdivisions of the mammalian POA also express markers for GABAergic neurons (Sherin, Elmquist, Torrealba, & Saper, 1998; Wu et al., 2014), we also used double-label fluorescent IHC to determine whether Gal-ir neurons in the POA-AH coexpress GABA.

This is, to our knowledge, the first description of Gal-ir systems in the brain of a vertebrate with alternative male reproductive morphs as well as in a vocal teleost. Somewhat surprisingly, given the convergence between type II males and females in many characters including vocal ones (see earlier references), we find that females differ dramatically from both male morphs in the number of galanin-expressing somata and in the distribution of fibers, especially in brainstem vocal-acoustic nuclei and other sensory integration sites. Some of these regions also differ, though less extensively, between the male morphs. This pattern extends to a greater density of Gal-ir fibers among males in all of the major vocal and auditory nuclei. Colabeling with either an AVP or OT antibody indicates that Gal neurons are a primarily nonapeptidergic population, while colabel for GABA shows a greater fraction, but still a minority, of Gal neurons that coexpress the transmitter. More broadly, the results are consistent with a role for Gal in regulating brain states that require the coordination of neuroendocrine, sensory, and motor systems especially in male-specific behaviors.

2 | MATERIALS AND METHODS

2.1 | Tissue collection

Twenty-nine adult midshipman (11 type I males, 24.26–189.86 g, 13.3–23.2 cm standard length; nine type II males, 2.78–15.33 g, 6.5–11.4 cm; nine females, 15.87–71.14 g, 11.7–18.5 cm) were collected from nests in June–July of 2016 and May–August of 2018 in Washington and California, and shipped overnight to Cornell University. Fish were held in environmental control rooms at 16–18°C on a 15:9 hr light:dark cycle until sacrifice. Midshipman adult morphs are distinguishable by a number of somatic traits. Morph type was determined at

sacrifice on the basis of gonad type and size and swim bladder sonic muscle size and color (Bass & Marchaterre, 1989; Brantley & Bass, 1994).

Animals were deeply anesthetized in 0.025% benzocaine (Sigma-Aldrich, St. Louis, MO), then perfused with ice cold teleost ringer's solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for label of Gal with AVP and OT or 4% paraformaldehyde+0.1% glutaraldehyde in 0.1 M PB for label of Gal with GABA. Following perfusion, brains were removed and post-fixed for 1 hr at 4°C, then transferred to 0.1 M PB for storage at 4°C. Prior to sectioning, brains were cryoprotected in 25% sucrose in 0.1 M PB overnight at 4°C, then frozen in Tissue Plus O.C.T. compound (TissueTek, Torrance, CA) at –80°C. Brains were sectioned at 25 μ m in four series and thaw mounted onto Superfrost Plus slides (ThermoFisher Scientific, Waltham, MA). Slides dried overnight at room temperature, then were stored at –80°C. All methods for collection of tissue were approved by the Cornell University Institutional Animal Care and Use Committee.

2.2 | Immunohistochemistry

Prior to labeling, slides were returned to room temperature and allowed to dry. Slides were washed three times for 10 min in phosphate buffered saline (PBS), followed by a 2 hr incubation in blocking solution of 0.2% bovine serum albumen (BSA, Sigma Aldrich, St. Louis, MO), 0.3% Triton-X 100 (Sigma Aldrich), and 10% normal goat serum (NGS, ThermoFisher) in PBS, then 18 hr in primary antibody (Table 1) in blocking solution at room temperature. Following primary antibody incubation, slides were washed three times in PBS, then incubated 2 hr in secondary antibody (Table 1) in 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, then cover slipped with ProLong Gold with DAPI (ThermoFisher). After cover slipping, slides were allowed to dry at room temperature overnight, then edges were sealed with nail polish. Slides were stored at 4°C.

2.3 | Primary antibody characterization

2.3.1 | Galanin (Gal)

We detected Gal using a custom antibody raised against midshipman Gal peptide (Table 2). Midshipman Gal precursor sequence was obtained from RNA-sequencing data of the midshipman POA-AH (Tripp et al., 2018) and then translated to amino acid sequence. Gal peptide was synthesized and a custom antibody raised in guinea pig and affinity purified by Pocono Rabbit Farm & Laboratory (PRF&L, Canandensis, PA). Antibody specificity was confirmed by IHC as described above with primary antibody omitted or preadsorbed with 50 μ M midshipman Gal (PRF&L) for 4 hr at room temperature.

2.3.2 | Arginine-vasotocin

We detected AVT using a polyclonal antibody raised in rabbit against AVP (Sigma) conjugated to thyroglobulin (AB1565, Millipore). The manufacturer reports less than 1% cross-reactivity with OT. Specificity and

TABLE 1 List of antibodies used in this study

Antibody name	Immunogen	Host	Antibody type	Dilution	Manufacturer	Catalogue number, RRID
<i>Primary antibodies</i>						
Galanin	Midshipman galanin conjugated to keyhole limpet hemocyanin	Guinea pig	Polyclonal	1:250	Pocono Rabbit Farm & Laboratory	Custom antibody, RRID:AB_2783794
Arginine vasopressin	Arginine vasopressin conjugated to thyroglobulin	Rabbit	Polyclonal	1:500	Millipore	AB1565, RRID:AB_90782
Oxytocin	Synthetic oxytocin conjugated to thyroglobulin	Rabbit	Polyclonal	1:500	Millipore	AB911, RRID:AB_2157629
GABA	GABA conjugated to bovine serum albumen	Rabbit	Polyclonal	1:1,000	Sigma-Aldrich	A2052, RRID:AB_477652
<i>Secondary antibodies</i>						
Goat anti-guinea pig Alexa Fluor 488	Gamma immunoglobins heavy and light chains	Goat	Polyclonal	1:1,000	ThermoFisher Scientific	A-11073, RRID:AB_142018
Donkey anti-rabbit Alexa Fluor 568	Gamma immunoglobulin	Donkey	Polyclonal	1:200	ThermoFisher Scientific	A10042, RRID:AB_2534017
Donkey anti-rabbit Alexa Fluor 680	Gamma immunoglobulin	Donkey	Polyclonal	1:200	ThermoFisher Scientific	A10043, RRID:AB_2534018

Abbreviation: GABA, γ -aminobutyric acid.

detection of AVT in midshipman brain was confirmed by IHC with primary antibody omitted or preadsorbed with 50 μ M AVT (Bachem) for 4 hr at room temperature.

2.3.3 | Isotocin

IT was detected using a polyclonal antibody raised in rabbit against synthetic OT (Sigma) conjugated to thyroglobulin (AB911, Millipore). The manufacturer reports less than 1% cross-reactivity with AVP. Specificity and detection of IT in midshipman was confirmed by IHC with primary antibody omitted or preadsorbed with 50 μ M or 100 μ M IT (Bachem) for 4 hr at room temperature.

2.3.4 | γ -Aminobutyric acid

To detect GABA, we used a polyclonal antibody raised in rabbit against GABA conjugated to BSA (A2052, Sigma). The manufacturer reports positive binding with GABA and GABA conjugated to keyhole limpet hemocyanin and negative binding to BSA in a dot blot assay. This antibody has previously been used in fish (Bai, Garver, Hukriede, & Burton, 2007; Dunn et al., 2016; Jusuf et al., 2011), and elimination of label following preadsorption of antibody with GABA has been reported in a broad range of species (Chalazonitis et al., 2009; Iwano et al., 2010; Webber, Thomson, Buckland-Nicks, Croll, & Wyeth, 2017). Specificity in midshipman was confirmed by IHC with primary antibody omitted.

2.4 | Imaging and analysis

Patterns of Gal expression were identified using a Zeiss Axioskop 2 Plus light microscope by an observer blinded to morph and sex of

the fish. Assessment of intensity of label was assigned to one of four categories: absent, sparse, moderate, or dense, and was confirmed by a second observer also blinded to morph and sex of the fish. Images of representative sections were collected using a Zeiss LSM 880 confocal microscope (Cornell University Biotechnology Resource Center Imaging Facility, NIH S10OD018516) with Zen software (v2.1). Whole section images were collected at 10 \times using tile scanning with a medium (0.70) threshold for stitching. High magnification images were collected at 40 \times with a 10-level z-stack (5 μ m optical section, 2.5 μ m step).

Images for analysis of coexpression of Gal and AVT, IT, or GABA were collected using the Zeiss confocal microscope at 20 \times with a 10-level z-stack (5 μ m optical section, 2.5 μ m step) for each section through the POA-AH. Tile scanning with 20% overlap and medium stitching threshold was used when necessary to capture the entire POA-AH in an image. Identification of coexpression of AVT, IT, or GABA in Gal neurons was done using FIJI (Schindelin et al., 2012) by an observer blind to the morph of each fish. Green and red channels were z-projected separately using the maximum intensity function. Somata of Gal neurons were first identified in the green channel image using two criteria: (1) a clearly defined perimeter and (2) an identifiable nucleus and/or neurite. Then, the observer determined whether the Gal neuron was also labeled by the AVP, OT, or GABA antibody by switching to the red channel image and identifying whether above-background signal was present within the boundaries of the Gal-ir soma. Images were cropped and re-sized for figures using Adobe Photoshop CS6 (Adobe Systems Incorporated, San Jose, CA). Red images were converted to magenta for figures using FIJI.

For comparison of Gal-ir cell numbers in the POA-AH, somata were identified and counted using the above criteria on 20 \times images from a single series of four (see above) from six individuals of each

TABLE 2 Gal peptide sequences from several vertebrate lineages

Midshipman	G	W	T	L	N	S	A	G	Y	L	L	G	P	H	G	I	D	G	H	R	T	L	G	D	K	A	G	L	A				
Rainbow Trout ^a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	S	-	-	H	-	-	-	-				
Cichlid ^b	-	-	-	M	-	-	-	-	-	-	-	-	-	-	-	L	-	-	L	H	G	H	T	R	L	G	D	K	P	G	L	A	
Frog ^c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	S	F	N	-	-	H	-	-	-	-	-	-	-
Alligator ^d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	S	F	N	E	-	H	-	I	-	-	-	-	-
Chicken ^e	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	V	-	-	-	S	F	N	-	-	H	-	F	T	-	-	-	-
Pig ^f	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	-	-	-	-	S	F	H	-	-	Y	-	-	-	-	-	-	-
Human ^g	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	A	V	G	N	-	S	F	S	-	-	N	-	-	T	S	-	-	-

Note: Dashes indicate identity with midshipman sequence.

Abbreviation: GAL, galanin.

^a*Onchorynchus mykiss* (Anglade et al., 1994).

^b*Astatotilapia burtoni* (Hu et al., 2016).

^c*Rana ridibunda* (Chartrel, Wang, Fournier, Vaudry, & Conlon, 1995).

^d*Alligator mississippiensis* (Wang & Conlon, 1994).

^e*Gallus gallus domesticus* (Norberg, Sillard, Carlquist, Jörnvall, & Mutt, 1991).

^f*Sus scrofa domestica* (Tatemoto et al., 1983).

^g*Homo sapiens* (Schmidt et al., 1991).

morph, then multiplied by four to estimate the total cell count. For Abercrombie correction of cell counts (Abercrombie, 1946), diameters of somata were measured in FIJI at the level of the anterior commissure and at the level of the magnocellular nucleus of the preoptic area in order to account for size differences between parvocellular and magnocellular neurons. Average diameter was taken from 10 somata from each population, except when fewer than 10 somata were present, in which case the average was taken from all somata at that level. In cases where sections were lost or damaged, the number of somata present was estimated by taking the average of the two adjacent sections in the series. For statistical analyses, whole-POA-AH Abercrombie-corrected cell counts were adjusted for standard length, then compared using one way analysis of variance (ANOVA) with Tukey post hoc test for significance.

3 | RESULTS

3.1 | Overview of Gal-ir somata and fibers

Custom Gal antibody robustly and specifically labeled somata and fibers. We map the general pattern of Gal-ir somata and fibers for type I males that have been the subject of most prior neuroanatomical and neurophysiological studies of midshipman (Figures 1–4). Although both male morphs have robust labeling throughout the brain, type II males have reduced intensity of label in some regions (Figure 5, Table 3). In contrast, females have much reduced or absent label in regions where Gal-ir label was robust in both male morphs (Figure 5, Table 3). As reported below, these polymorphic patterns are especially noticeable in the midbrain (Figure 5g–i) and hindbrain (Figure 5j–l). A complete list of regions showing sexual polymorphism is presented in Table 3. Figure 1a indicates the approximate levels along the rostral-caudal axis that are illustrated in Figures 2–4 (levels for 2c, 3e, and 4e correspond to levels shown in Figure 5; see legend for Figure 1), and includes representative Nissl-stained sections from non-experimental material that provide more cytoarchitectural detail of the regions illustrated in Figures 2–4. The nomenclature used here largely follows that used in prior publications on midshipman fish (e.g., Feng et al., 2019) and zebrafish (Wullmann, Rupp, & Reichert, 1996).

3.1.1 | Gal-ir somata

Gal-ir label of fibers and somata extends throughout the subpallium (area ventralis of the telencephalon, V) into the preoptic area (Figure 2a–k). Robustly labeled Gal-ir somata first appear and are most abundant in the anterior parvocellular nucleus of the preoptic area (PPa) with additional cells in the posterior parvocellular (PPp) and magnocellular (PM) nuclei (Figure 2c,f,g,i–k). Females consistently have significantly fewer somata labeled than either male morph (Figures 5a–f and 6). One-way ANOVA comparison of Gal-ir cell counts shows a significant difference ($F_{[2,15]} = 4.937, p = .0225$) for total number of Abercrombie-corrected (Abercrombie, 1946), length adjusted Gal-ir neurons. Tukey post hoc test shows that both type I

TABLE 3 Sexual polymorphisms in Gal-ir fiber distribution

Anatomical location	Type I male	Type II male	Female
<i>Telencephalon</i>			
Medial zone of area dorsalis (Dm)	+	+	–
Lateral zone of area dorsalis (DI)	+	+	–
Dorsal zone of area dorsalis (Dd)	+	+	–
<i>Diencephalon</i>			
Habenula (Hb)	+	+	–
<i>Mesencephalon</i>			
Periaqueductal gray (PAG) VOCAL	+++	+++	+
Mesencephalic tectum (TeM) VISUAL	++	++	–
Periventricular layer of torus semicircularis (TSp) AUDITORY	+	+	–
Deep layer of torus semicircularis (TSd) AUDITORY, VOCAL, LATERAL LINE	+++	+++	–
Nucleus of the lateral lemniscus (nLL) AUDITORY	+++	++	–
Rostral tegmental nucleus (RT) VOCAL	+	+	–
Isthmal auditory-vocal nucleus (IS) AUDITORY, VOCAL	+++	+++	+
Paralemniscal midbrain tegmentum (PL) VOCAL	+++	++	–
Isthmal paraventricular nucleus (IP) VOCAL	++	++	+
<i>Rhombencephalon</i>			
Reticular formation (level of VMN)	+++	++	+
Vocal prepacemaker nucleus (VPP) VOCAL	+	+	–
Vocal pacemaker nucleus (VPN) VOCAL	+	+	–
Vocal motor nucleus (VMN) VOCAL	+	+	–

Note: Symbols indicate density of Gal-ir fibers in each anatomical location for each morph. +++ indicates dense, ++ indicates moderate, and + indicates sparse, – indicates absence of Gal-ir fibers. Abbreviation: GAL-ir, galanin-immunoreactivity.

($p = .0458928$) and type II ($p = .0343761$) males have significantly more Gal-ir neurons than females, but do not differ from each other ($p = .9875526$). Scattered, less strongly labeled Gal-ir somata are also detected in the hypothalamus of all morphs in the anterior tuberal (AT; Figure 3b,c) and the lateral hypothalamic (LH; Figure 3d,e) nuclei.

3.1.2 | Forebrain and pituitary Gal-ir fibers

Sparse Gal-ir fibers are found in the olfactory bulb of all morphs (not shown). In the pallium (area dorsalis of the telencephalon), sporadic fibers are also present in the medial, central, lateral, and dorsal zones (Dm, Dc, DI, Dd; Figure 2a–c,g,i), with only type I males showing label in these regions consistently. The density of Gal-ir fiber systems increases in the subpallium with all morphs showing moderate label in the ventral and dorsal nuclei (Vv, Vd; Figure 2a–e) that becomes dense closer to the anterior commissure (ac; Figure 2b,e). Fiber density is especially high in the supracommissural nucleus (Vs, Figure 2c), but quite sparse in regions caudal to the anterior commissure (e.g., the postcommissural nucleus, Vp, Figure 2g).

In the POA, dense fibers are seen within and lateral to the PPa and PPp (Figure 2b,c,g,i), as well as in the neuropil lateral to PM and in the preoptico-hypophysial tract comprised of axons that innervate the pituitary (PHT, Figure 2g,i; also see Foran & Bass, 1998). Sparse fibers are also seen among the cells of PM (Figure 2j,k). Gal-ir fibers are found in moderate density within the pituitary of all morphs (Figure 2h). Elsewhere in the anterior hypothalamus, fiber label is present in the vocally active ventral tuberal nucleus that innervates vocal midbrain regions (Goodson & Bass, 2000a, 2002). Label in the ventral tuberal nucleus is dense at rostral levels (vT, Figure 2i) that becomes relatively sparse at more caudal levels. Dense label also surrounds the suprachiasmatic nucleus (SCN, Figure 2i). Sparse and dense labeling of fibers is apparent in the rostral and caudal levels, respectively, of the auditory-recipient and vocally active anterior tuberal nucleus (AT; Figure 3a–d) (see Bass, Bodnar, & Marchaterre, 2000; Goodson & Bass, 2000b) and in the lateral hypothalamus (LH; Figure 3b,d,e).

Sparse labeling of Gal-ir fibers is seen in the habenula for both type I and II males (Hb, Figure 3a), but is absent in females (Table 3). Moderately dense label in the dorsal thalamus is in the central posterior nucleus (CP, Figure 3a,b) that receives auditory input from the midbrain (Bass et al., 2000). There is a large density of fibers occurring in the ventral and dorsal portions of the retinal-recipient periventricular pretectal nucleus (PPv, PPd; Figure 3a,b). Additional fibers ranging from sparse to dense with a large degree of individual differences are found in the posterior tuberal nucleus and the periventricular nucleus of the posterior tuberculum (TP, TPp; Figure 3a,b,d), and in the dorsal, ventral, and central (not shown) zones of the periventricular hypothalamus (Hd, Hv; Figure 3a,b,d; see panel 3e in Figure 1 for Hc location).

3.1.3 | Midbrain Gal-ir fibers

Some of the most dramatic examples of sexual polymorphisms in Gal-ir fiber distributions are in the midbrain, inclusive of vocal, auditory, lateral line, and visual regions (Table 3). A prime example of this is the periaqueductal gray (PAG) that gates descending input from vocally active sites in the ventral tuberal nucleus to the hindbrain vocal pattern generator that receives direct PAG input (Goodson & Bass, 2000a, 2002; Kittelberger et al., 2006; Kittelberger & Bass, 2013). A very dense field of labeled processes in the PAG of males (Figure 3d,f,h) is almost entirely absent in females (Figure 5g–i). Males show sparse

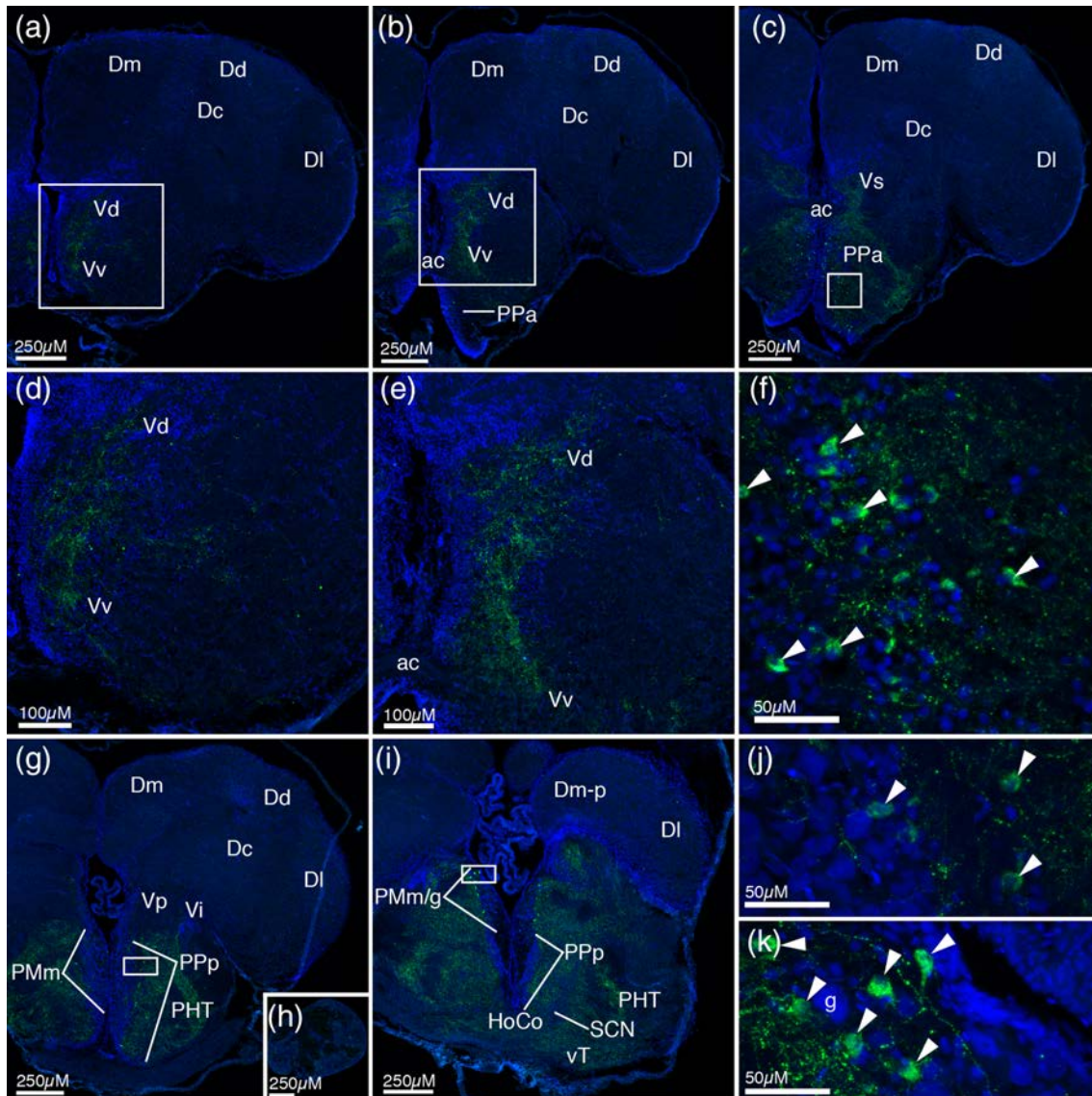


FIGURE 2 Forebrain galanin (Gal) distribution. Gal-immunoreactivity (green) in the telencephalon and preoptic area of the midshipman brain with DAPI nuclear stain (blue). Images from a representative type I male taken at the approximate levels indicated in Figure 1a. Also see Figure 1b for representative Nissl-stained sections from nonexperimental material that provide cytoarchitectural detail for illustrated regions. (a) Gal label appears in moderate density beginning in dorsal (Vd) and ventral (Vv) nuclei of the ventral telencephalon. Box indicates region shown in (d). (b) Density of Gal label increases at the level of the anterior commissure (ac), where the anterior parvocellular nucleus of the preoptic area (PPa) begins; sparse fibers also appear in dorsal telencephalon. Box indicates region shown in (e). (c) Gal-ir somata are most abundant in PPa. Box indicates region shown at higher magnification in (f), arrows indicate Gal-ir somata. (g) Somata are also present throughout the posterior parvocellular nucleus of the preoptic area (PPp, small cells), which runs lateral and ventral to the magnocellular neurons within the magnocellular nucleus of the preoptic area (PMm, larger cells) at this level. Box in (g) shows region containing Gal-ir cells in PPp shown at higher magnification in (j). Lines indicate dorsal-ventral extent of PMm and PPp. (h) Gal-ir fibers in the pituitary (i) Gal-ir somata continue into magnocellular preoptic area (PM). Only PMm cells express Gal, though they are interspersed with even larger gigantocellular neurons within the PM (PMg). Fibers are sparse within PM, but dense fibers are present lateral to PM and in the preoptico-hypophyseal tract (PHT). Box indicates region depicted at higher magnification in (k). (k) Gal-ir PMm neurons. Letter g indicates a gigantocellular cell that is not labeled for Gal. Also see list of abbreviations [Color figure can be viewed at wileyonlinelibrary.com]

fibers in the periventricular layer of the auditory-encoding division (see Bodnar & Bass, 1997) of the torus semicircularis (TSp; Figure 3f,i). Label greatly increases in density in the deep layer of the torus (TSd; Figure 3f,i) that receives input from the more dorsal auditory torus (Bass et al., 2000; also see Bass, Bodnar, & Marchaterre, 2001 for closely related toadfish) and is a vocally active site (Feng & Bass, 2014).

Gal-ir label is absent in the torus semicircularis of females (Figure 5i), with the exception of one fish that had very sparse fibers in the deep layer. Additionally, both male morphs, but not females, have two clearly defined layers of Gal-ir fibers in the stratum album centrale (SAC) and stratum griseum centrale (SGC) near its border with the stratum album centrale in the retinal-recipient tectum

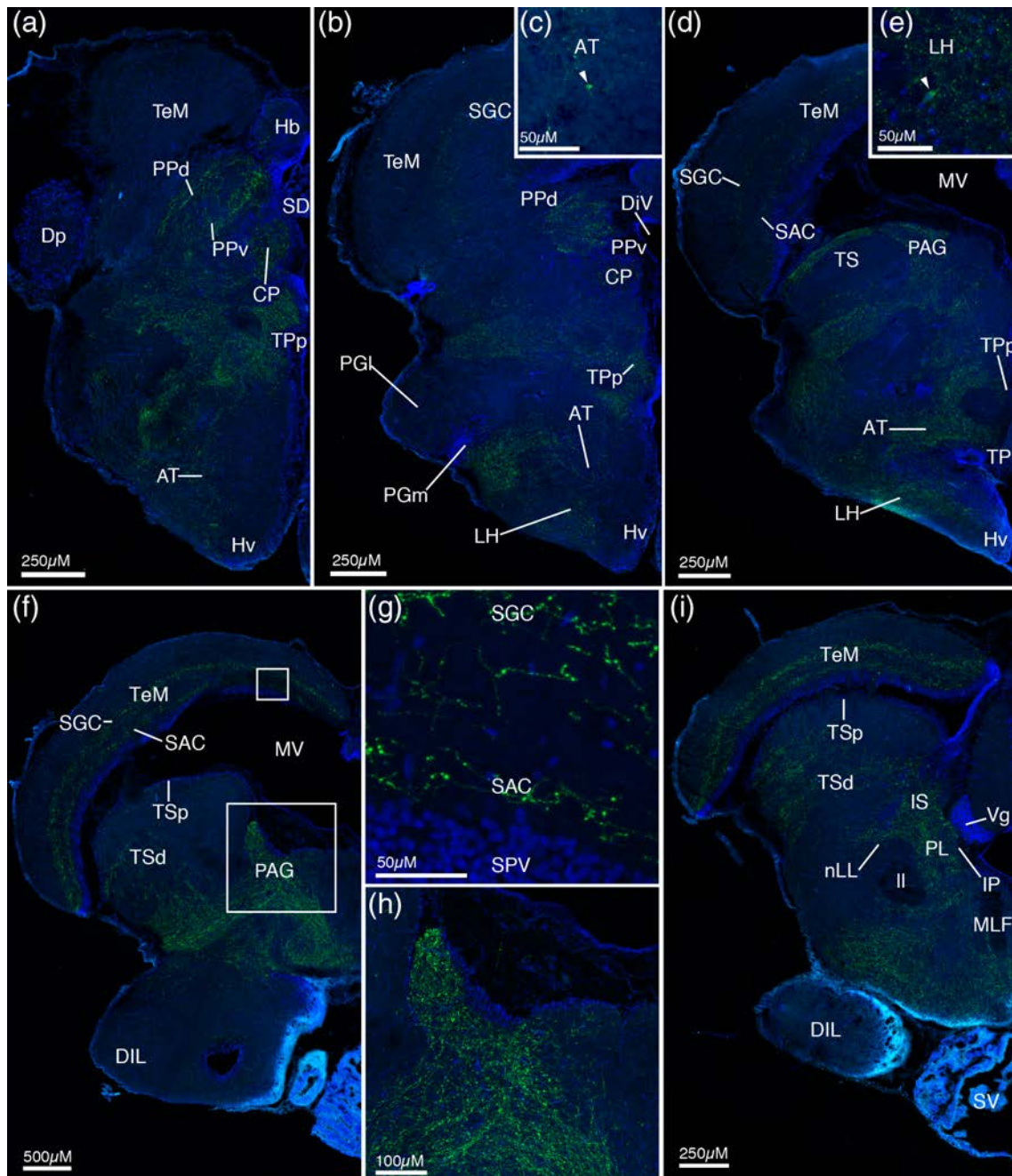


FIGURE 3 Diencephalon and midbrain galanin (Gal) expression. Gal-immunoreactivity (green) in the diencephalon and midbrain of the midshipman with DAPI nuclear stain (blue). Images from a representative type I male taken at the approximate levels indicated in Figure 1a. Also see Figure 1b for representative Nissl-stained sections from nonexperimental material that provide cytoarchitectural detail for illustrated regions. (a) Fibers present in several diencephalic nuclei, including sparse fibers in rostral end of anterior tubular nucleus (AT) and denser fibers within pretectal nuclei (PPd/PPv), central posterior (CP), and periventricular nucleus of the posterior tuberculum (TPp). (b) Fibers continue more caudally, and sparse fibers appear at rostral level of lateral hypothalamus (LH). (c) Gal-ir neuron in AT at similar level as (b). (d) Fiber densities at the level of anterior midbrain. Fibers apparent in stratum album centrale (SAC) and stratum griseum centrale (SGC) layers of the mesencephalic tectum (TeM) and rostralmost level of torus semicircularis (TS) and periaqueductal gray (PAG). Fiber density highest in AT and LH at this level. (e) Gal-ir neuron in LH at similar level as (d). (f) Continuing fiber label in the midbrain including tectum (TeM), periaqueductal gray (PAG) and torus semicircularis (TSp/TSd), and caudal diencephalon. Boxes indicate regions shown at higher magnification in (g) and (h) for TeM and PAG, respectively. (g) Gal-ir fibers present in SAC and SGC layers of TeM. (h) PAG contains some of the most dense Gal-ir fibers in the male brain. (i) Gal-ir fibers continue through the caudal midbrain, including nuclei of the auditory tegmentum: Paralemniscal nucleus (PL), nucleus of the lateral lemniscus (nLL), and isthmal auditory-vocal nucleus (IS). Also see list of abbreviations [Color figure can be viewed at wileyonlinelibrary.com]

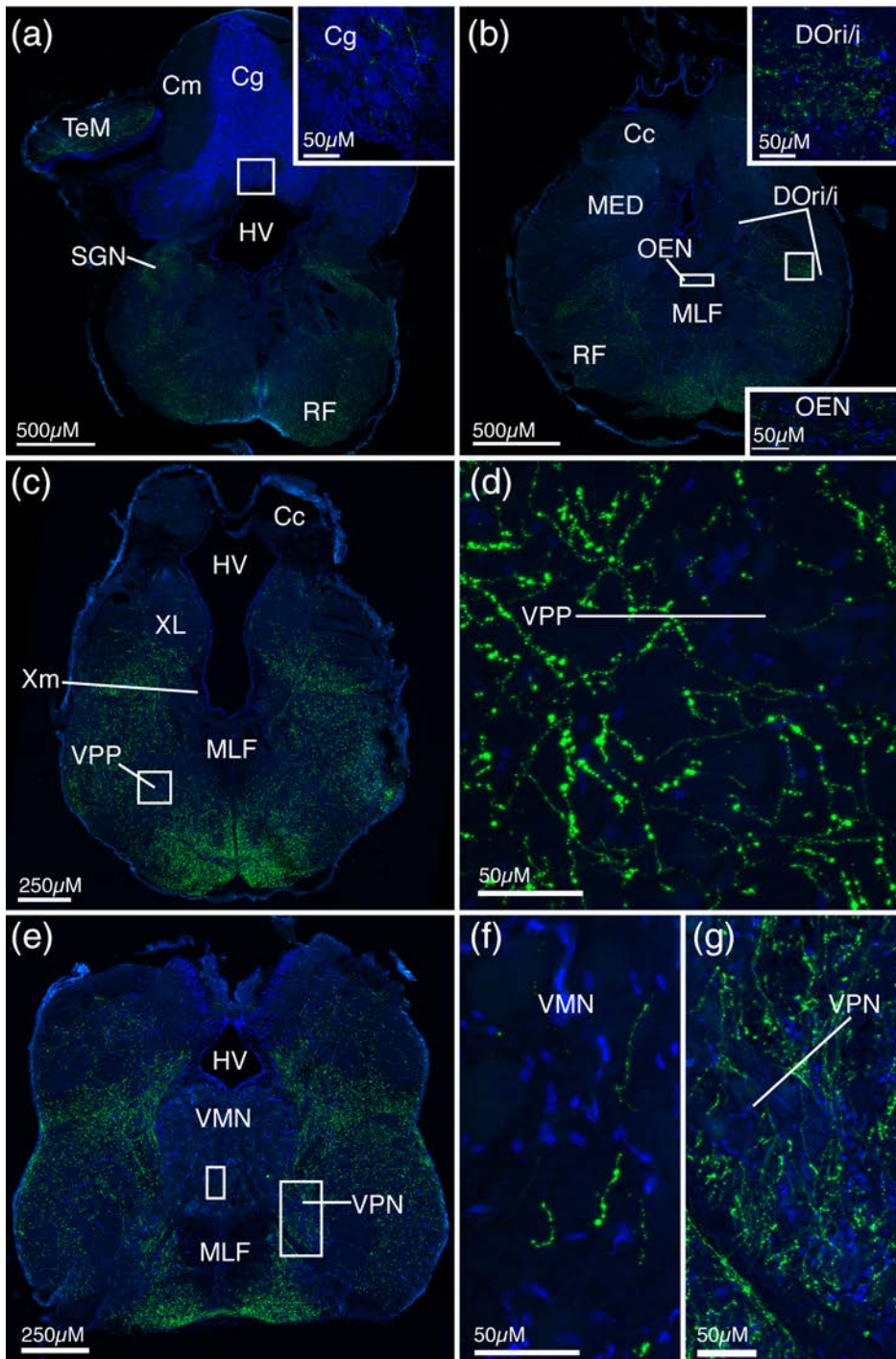


FIGURE 4 Hindbrain galanin (Gal) expression. Gal-immunoreactivity (green) in the hindbrain of the midshipman with DAPI nuclear stain (blue). Images from representative type I males taken at the levels indicated in Figure 1a. Also see Figure 1b for representative Nissl-stained sections from non-experimental material that provide cytoarchitectural detail for illustrated regions. (a) Sparse Gal-ir fibers in the granule cell layer of the cerebellum (Cg) with dense fibers throughout the reticular formation (RF). Box indicates region shown at higher magnification in insert. (b) Fibers in octaval efferent nucleus (OEN) (higher magnification in lower insert indicated by box); label is dense at this level in rostral-intermediate division of the descending octaval nucleus (DORI, higher magnification in upper insert indicated by box). Fibers are found in the vocal-motor central pattern generator of males only, including vocal prepacemaker (VPP) (c,d), pacemaker (VPN) (e,g), and vocal motor (VMN) nuclei (e,f). Fibers are sparse within nuclei, but highly dense in the neuropil immediately surrounding VPP, and VPN/VMN. Boxes in (c) and (e) indicate regions shown at higher magnification in (d), (f), and (g). Images in (c–g) taken from a fish collected following humming and colabeled with phosphorylated S6 protein, a neural activity marker (Knight et al., 2012), which positively labeled and aided identification of VPP and VPN (J. A. Tripp, I. Salas-Allende & A. H. Bass; unpublished observations). Also see list of abbreviations [Color figure can be viewed at wileyonlinelibrary.com]

(Figures 3b,d,f,g,i and 5g–i) that also receives input from the auditory torus semicircularis and PAG (Bass et al., 2000; Kittelberger & Bass, 2013).

Type I males have dense Gal-ir fibers distributed within the nucleus of the lateral lemniscus, the isthmal auditory-vocal nucleus and the paralemniscal region adjacent to the lateral lemniscus (nLL, IS, PL; Figure 3i), which were present in moderate density in type II males. Females lack fibers in nucleus of the lateral lemniscus and paralemniscal region and have only sparse to moderate expression of Gal-ir fibers in the isthmal auditory-vocal nucleus. All males, but not

females, have sparse fibers within the rostral tegmental nucleus (not shown, see Pengra et al., 2018). Finally, the isthmal paraventricular nucleus has a moderate density of fibers in males (IP, Figure 3i) that was reduced but present in females. Both neurophysiological and neuroanatomical studies show the involvement of the nucleus of the lateral lemniscus, isthmal auditory-vocal nucleus, rostral tegmental nucleus, paralemniscal region and isthmal paraventricular nucleus in the central vocal-auditory network of midshipman (Bass, March-atterre, & Baker, 1994; Feng & Bass, 2014; Goodson & Bass, 2000b; Kittelberger & Bass, 2013).

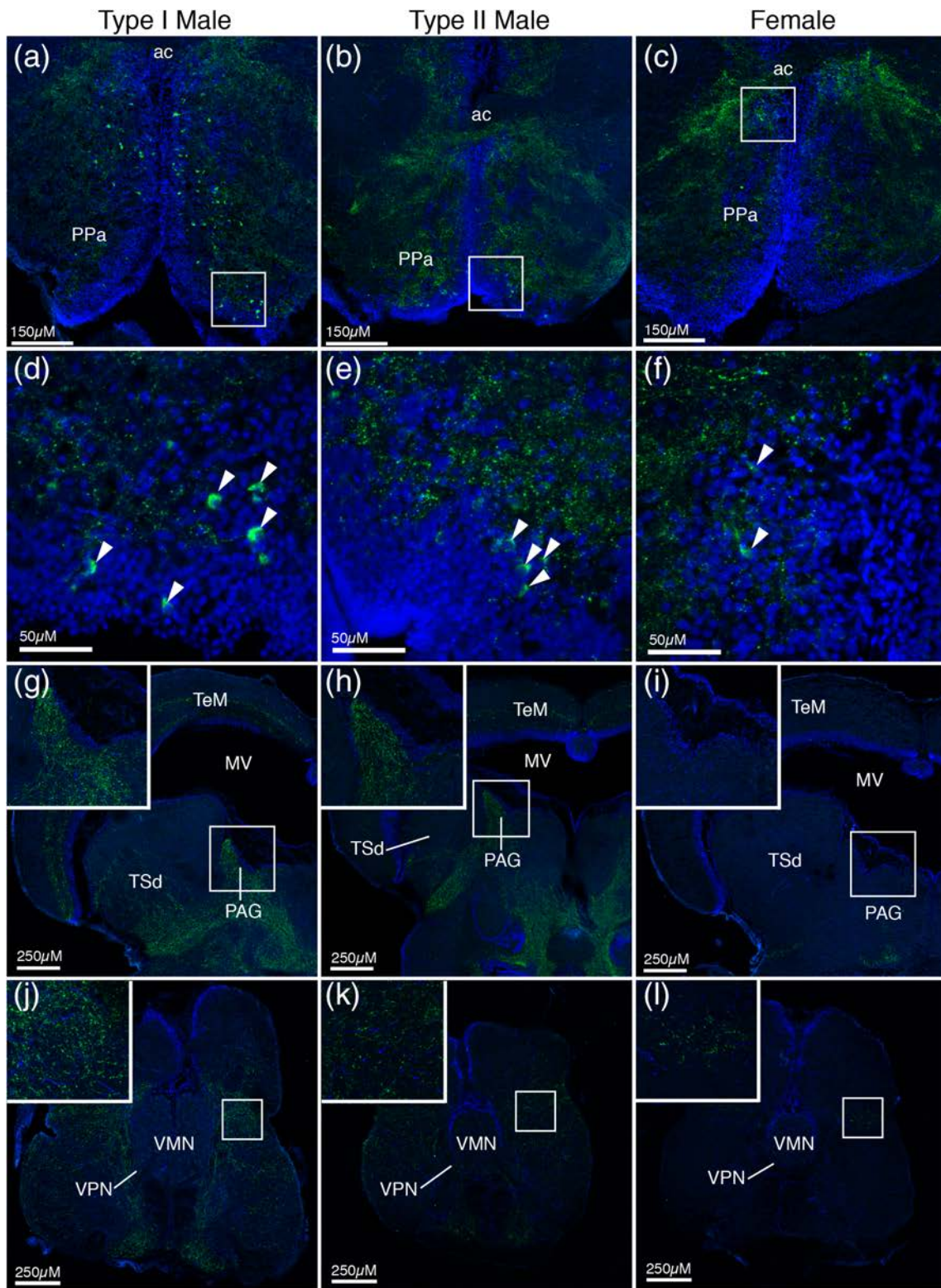


FIGURE 5 Sex and male morph differences in galanin (Gal)-ir distribution in the midshipman brain. Gal-immunoreactivity (green) with DAPI nuclear stain (blue). Gal-ir neurons found in anterior parvocellular preoptic area (PPa) of all morphs (a–f), but much less abundant in females (c, f). Males also have much more dense expression of Gal-ir fibers in midbrain tectum (TeM), periaqueductal gray (PAG), and torus semicircularis (TS) (g, h) compared to females (i); type II label is also reduced compared to type I males. In the hindbrain, type I males have sparse Gal-ir fibers within the pacemaker (VPN) and vocal motor (VMN) nuclei and dense expression surrounding these nuclei (j), which is reduced in type II males (k). Females show no Gal-ir fibers within VPN or VMN and greatly reduced expression of fibers in the surrounding hindbrain compared to males (l). Boxes in (a–c) indicate regions shown at higher magnification in (d–f) and boxes in (g–l) indicate regions shown in inserts. Also see list of abbreviations [Color figure can be viewed at wileyonlinelibrary.com]

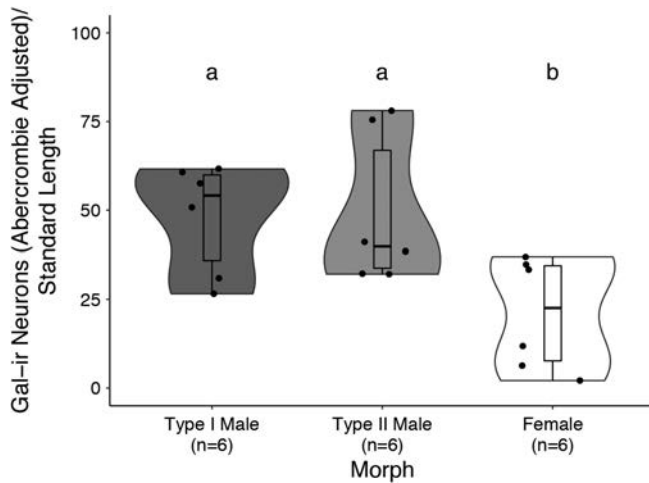


FIGURE 6 Male midshipman have significantly more galanin (Gal) neurons in the preoptic area-anterior hypothalamus than females. Violin plots showing the total number of Gal-ir neurons in the preoptic area of type I male, type II male, and female midshipman after Abercrombie correction (Abercrombie, 1946) and adjustment for standard length. Width of each plot represents the probability density of the data. Lower and upper bounds of boxes indicate first and third quartiles, thick line within the box indicates median value, and whiskers extend to minimum and maximum values. Dots represent individual data points from each animal. One way ANOVA $F_{(2,15)} = 4.937, p = .0225$. Groups labeled with different letters significantly differed from one another by Tukey post hoc comparisons. ANOVA, analysis of variance

3.1.4 | Hindbrain Gal-ir fibers

Fibers are distributed in the secondary gustatory nucleus (SGN, Figure 4a) and throughout the reticular formation (RF, Figure 4a-c) at moderate to high density in males and females. In the cerebellum, Gal-ir fibers are in low density within the granule cell layer of the corpus of males and females (Cg, Figure 4a). Both male morphs and the brains of one of three females show labeled fibers in the octaval efferent nucleus (OEN, Figure 4b) that relays the temporal code for sound duration from the vocal pattern generator directly to the inner ear and lateral line organs (Chagnaud & Bass, 2013). Finally, Gal-ir label is present in all male brains, though sparse, throughout the vocal central pattern generator circuit that includes prepacemaker (VPP), pacemaker (VPN), and vocal motor (VMN) nuclei (Figure 4c-g). Gal-ir fibers are absent in the vocal central pattern generator in females (Figure 5j-l). At this same level, Gal-ir fibers are especially dense in type I males in an area immediately lateral to the vocal motor nucleus, with label being somewhat reduced in this region in type II males and absent in females (inserts, Figure 5j-l; Table 3).

3.2 | Molecular characterization of galanin neurons

In order to characterize the peptides and transmitters that may be coreleased from POA-AH Gal-ir neurons, we colabeled midshipman POA-AH tissue for Gal and the nonapeptides AVT and IT, using antibodies raised against AVP and OT, respectively, or GABA. In general,

levels of coexpression for these peptides and transmitter in Gal-ir neurons were similar across morphs. Because somata are relatively infrequent and less strongly labeled in the anterior tuberal nucleus and lateral hypothalamus (Figure 3c,e), coexpression was not analyzed in these regions.

3.2.1 | Galanin coexpression with nonapeptides

Antibodies raised against AVP and OT label similar POA-AH populations as previously reported for the plainfin midshipman (Foran & Bass, 1998; Goodson et al., 2003; Goodson & Bass, 2000b). Anti-AVP antibody robustly labels somata and fibers, and label is eliminated following preadsorption with 50 μ M of AVT (AVP homologue in fish that differs by one amino acid; Bentley, 1998) or by omission of primary antibody. We did not detect coexpression of AVP in Gal-ir neurons (Figure 7a-c) in any morph ($n = 3$ fish per morph; type I males, 735 Gal-ir cells; type II males, 324 cells; females, 91 cells).

Anti-OT antibody robustly labels somata and fibers. Label is greatly reduced in a concentration-dependent manner following preadsorption with 50 μ M or 100 μ M IT (OT homologue in fish that differs by two amino acids; Bentley, 1998) and eliminated by omission of primary antibody. Coexpression of OT in Gal-ir neurons is exceedingly rare (Figure 7d-f). In type I males ($n = 3$ fish, 978 total Gal-ir neurons cells), just two Gal-ir neurons, one each from two animals, also had detectable OT-ir expression. In type II males ($n = 3$ fish, 474 total Gal-ir neurons cells), only a single Gal-ir neuron from one animal was also OT-ir. Finally, in females ($n = 3$ fish, 424 total Gal-ir neurons cells), no Gal-ir neurons had detectable OT-ir expression.

3.2.2 | Galanin coexpression with GABA

Antibody raised against GABA robustly labeled somata and fibers, and label is eliminated by omission of primary antibody. Colabel of GABA in POA-AH Gal-ir neurons (Figure 7g-i) is much greater than that observed for OT, and similar across all three midshipman adult morphs: $19.30 \pm 6.56\%$ (mean \pm SD) in type I males ($n = 3$ fish, 768 total Gal-ir cells), $19.54 \pm 3.77\%$ in type II males ($n = 3$ fish, 332 total Gal-ir cells), $25.90 \pm 8.18\%$ in females ($n = 3$ fish, 255 total Gal-ir cells).

4 | DISCUSSION

We report for midshipman fish collected during the breeding season, widespread Gal-ir fibers throughout the brain that apparently originate from relatively few cell somata, with stark differences between males and females. For both sexes, clusters of labeled somata are mainly located within the POA-AH, primarily in the parvocellular nuclei (PPa/PPp), though females have significantly fewer cells than males. In addition, Gal-ir fibers are prominent in the subpallium (area ventralis) and several diencephalic regions in both sexes. The general pattern of Gal-ir label is similar between the two male morphs, with type II males having reduced fiber density or intensity of label in some regions. However, compared to both male morphs, females

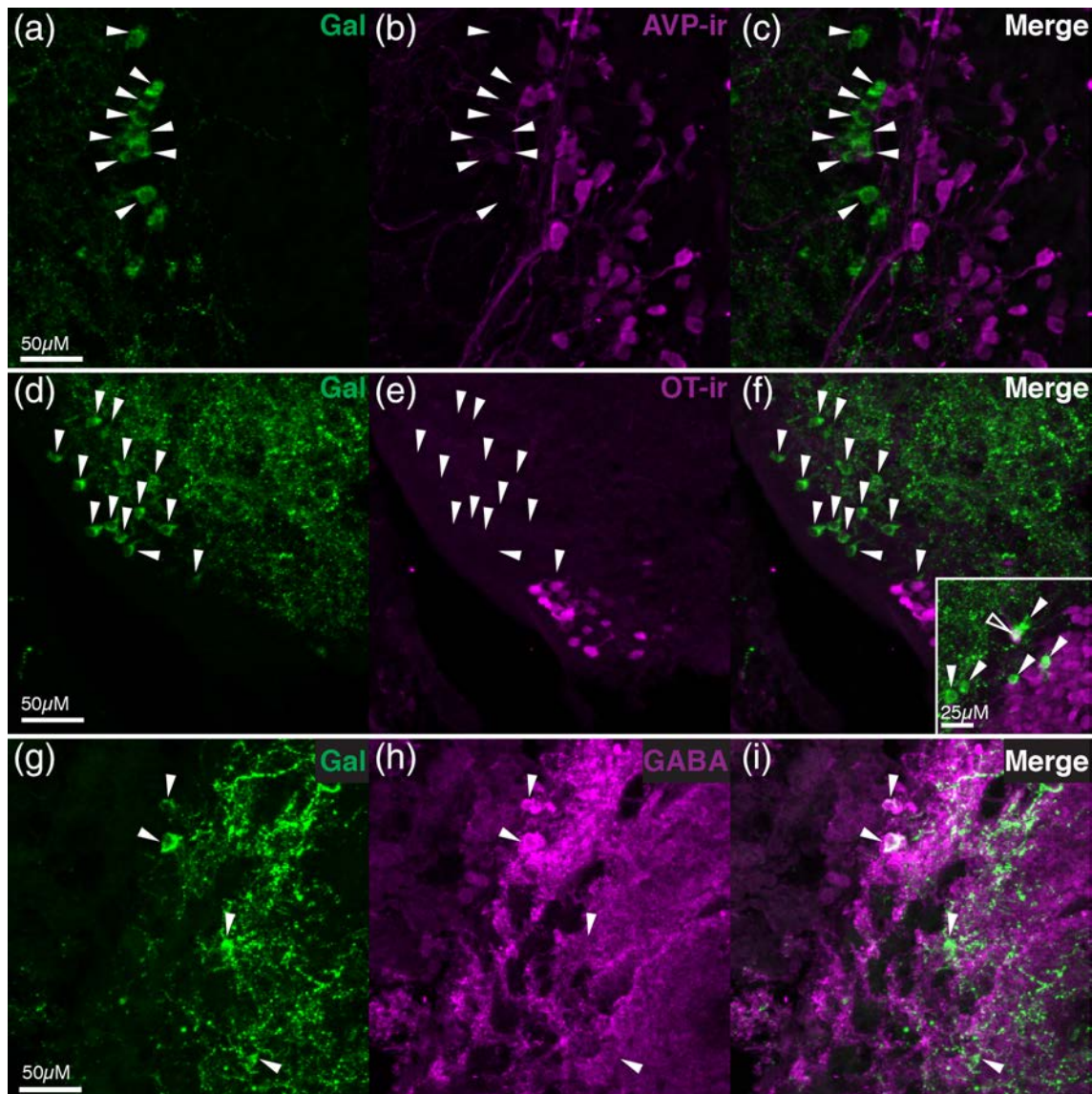


FIGURE 7 Neuropeptide coexpression with galanin (Gal) in the preoptic area-anterior hypothalamus of midshipman. (a–c) Galanin neurons do not coexpress arginine vasotocin (AVT) as documented using an antibody to its mammalian homologue, arginine vasopressin (AVP): (a) Gal-immunoreactive (ir) label (green), (b) AVP-ir label (magenta), (c) merged images showing that Gal and AVP/AVT are expressed in different cells. Image is from a type I male brain between the levels of Figure 2c.g. Arrows indicate position of Gal-ir somata here and in (d–i). (d–f) Very few galanin (Gal) neurons coexpress isotocin (IT) as documented using an antibody to its mammalian homologue, oxytocin (OT): (d) Gal-ir label (green), (e) OT-ir label (magenta), (f) merged images showing that Gal and OT/IT are expressed in different cells. Image is from a type I male brain at a similar level as Figure 2c. Arrows indicate position of Gal-ir somata. Insert in (f) shows rare example of Gal-ir neuron with OT-ir colabel (hollow arrow), surrounded by Gal-ir neurons that lack colabel (solid arrows). Insert image taken from type I male. In contrast to AVP/AVT and OT/IT, approximately 20–25% of Gal neurons also express GABA in both male morphs and females. Example image containing two Gal+/GABA+ neurons (upper two) and two Gal+/GABA– neurons (lower two). (g) Gal-ir label (green), (h) GABA-ir label (magenta), and (i) merged image. Image is from a type I male brain at similar level as Figure 2i [Color figure can be viewed at wileyonlinelibrary.com]

have greatly reduced or non-existent Gal-ir fiber distribution in select regions of the midbrain and hindbrain, including nuclei that are a part of vocal and auditory circuits.

4.1 | Gal-ir within vocal and auditory circuitry

Gal-ir label is prominent throughout the midshipman vocal-motor circuit. Somata are identified in three interconnected nuclei of the

forebrain vocal-acoustic complex (fVAC) (Goodson & Bass, 2002): PPa, PPp, and the anterior tuberal nucleus. Electrical stimulation of these regions results in vocal-motor output in midshipman (Goodson & Bass, 2000a, 2000b) and the closely related oyster toadfish (*Opsanus beta*) (Demski & Gerald, 1972; Fine & Perini, 1994). These regions contain a relatively high density of Gal-ir fibers, while another nucleus in the fVAC, the ventral tuberal nucleus that directly innervates the vocal PAG (Goodson & Bass, 2002; Kittelberger & Bass, 2013), also appears

to receive Gal input. Gal-ir fibers are also present in vocally active mid-brain sites (Kittelberger et al., 2006) in males but not females, with the PAG showing some of the most dense fibers in the male brain. Finally, scattered fibers are found throughout the male hindbrain vocal motor central pattern generator, which sets the duration, frequency, and final motor output, respectively, of midshipman calls (Bass & Baker, 1990; Chagnaud, Baker, & Bass, 2011; Chagnaud, Zee, Baker, & Bass, 2012).

Gal-ir fibers are also present in several auditory regions in all morphs. In the telencephalon, moderate to dense fibers were present in the supracommisural nucleus of the ventral telencephalon that has reciprocal connections with the central posterior nucleus, the auditory-recipient region in the dorsal thalamus (Goodson & Bass, 2002). In the diencephalon, both the central posterior nucleus and the periventricular nucleus of the posterior tuberculum that innervates both the hair cell epithelium of the auditory sacculae and the hindbrain octaval efferent nucleus that innervates the sacculae (Forlano, Kim, Krzymynska, & Sisneros, 2014; Perelmuter & Forlano, 2017), contain Gal-ir fibers in moderate density. Further posterior, in the midbrain, males and females differ in the Gal-ir innervation of several auditory regions. For males of both morphs these include Gal-ir fibers in the torus semicircularis, which receives ascending auditory and lateral line inputs (Bass et al., 2000, 2001; Weeg & Bass, 2000; the lateral line also encodes vocalizations—Weeg & Bass, 2002), nucleus of the lateral lemniscus, and isthmal auditory-vocal nucleus (Table 3). These fibers are mostly absent in females. Finally, sparse to moderate Gal-ir fibers are found in all males and one of three females in the hindbrain's octaval efferent nucleus that transmits a corollary discharge signal from the vocal central pattern generator to the inner ear and lateral line organs (Chagnaud & Bass, 2013; Weeg, Land, & Bass, 2005).

4.2 | Gal-ir within neuroendocrine nuclei and molecular characterization

As discussed above, the primary population of Gal-ir neurons in the male midshipman brain is found in the POA-AH, including PPa, PPp, and PM, while the number of neurons in these regions is significantly reduced in females. The POA-AH is a major site of neuroendocrine integration in the midshipman brain that has dense expression of both estrogen (Fergus & Bass, 2013; Forlano et al., 2005) and androgen (Forlano et al., 2010) receptors, as well as the enzyme aromatase that converts testosterone to estradiol (Forlano, Deitcher, Myers, & Bass, 2001). It also contains neurons that synthesize peptides including IT and AVT (Foran & Bass, 1998; Goodson et al., 2003; Goodson & Bass, 2000b) in addition to cells that synthesize Gal. Gal-ir fibers are also found within several forebrain neuroendocrine nuclei including throughout and in the neuropil lateral to the POA-AH, as well as in the ventral tuberal nucleus, anterior tuberal nucleus, lateral hypothalamus, and the suprachiasmatic nucleus. The density of fibers in PPa/PPp is slightly reduced in females (Table 3).

In all morphs, Gal-ir neurons in the POA-AH do not coexpress AVT, as indicated using an antibody to AVP. While Gal-ir and AVP-ir cell bodies are in close proximity in PPa and PPp with similar fiber projections, we found no evidence for coexpression of Gal and AVP.

Coexpression of Gal with OT is exceedingly rare. We found just two Gal-ir cells in type I males and one Gal-ir cell in a single type II male, which were also OT-ir. While Gal and nonapeptide-expressing neurons within the POA-AH appear to be separate populations, it should be noted that these cells are anatomically intermixed, and borders between populations are not always clear (see Figure 7f, inset for example). This is consistent with results from Atlantic salmon (*Salmo salar*) that show no coexpression of Gal with AVT or IT in POA neurons (Holmqvist & Ekstrom, 1991). However, one study of chickens found AVT expression in 100% of Gal-ir neurons in the supraoptic nucleus (Klein et al., 2006), which has proposed homology with the PPP of teleosts (Forlano & Cone, 2007; Herget, Wolf, Wullmann, & Ryu, 2014). Additionally, a recent study profiling single-cell expression profiles throughout the mouse preoptic region identifies one cluster of Gal-expressing cells that also express OT at a high level (Moffitt et al., 2018).

As with the nonapeptides, expression of GABA in Gal-ir neurons was consistent across morphs, with each showing coexpression in roughly one-fifth to one-quarter of Gal-ir cells. However, prior studies in mice indicate that a greater proportion of Gal neurons in the mammalian POA express GABA synthesis enzymes. In situ hybridization shows that approximately 90% of Gal-expressing neurons in the medial POA also express *Gad1*, a gene encoding the enzyme GAD involved in GABA synthesis (Wu et al., 2014). Additionally, IHC in mice shows the majority of Gal-ir neurons in the ventrolateral POA also express GAD protein, though no colabel was found in lateral POA or supraoptic nucleus (Sherin et al., 1998). Recent single-cell profiling of mouse preoptic region has also identified several Gal-expression cell clusters that express *gad1*, but also clusters that express markers for excitatory neurons (Moffitt et al., 2018). A caveat to these comparisons is that these studies investigated expression of a GABA-synthesizing enzyme, while our study labeled Gal-ir neurons for GABA itself. So while these GAD-expressing neurons have the capability to produce GABA (Levitan & Kaczmarek, 2002), it does not mean that they necessarily do so.

4.3 | Comparisons with other teleosts

As is the case for midshipman, Gal-expressing neurons have been largely identified in the POA-AH with fewer in other regions of the hypothalamus of all teleosts investigated to date. There is variation between species in that some have detectable Gal-ir somata in parvocellular nuclei only (Amano et al., 2009; Anglade et al., 1994; Batten et al., 1990; Olivereau & Olivereau, 1991; Yamamoto et al., 1992), while others also show somata in magnocellular populations (Cornbrooks & Parsons, 1991; Holmqvist & Ekstrom, 1991; Jadhao & Meyer, 2000; Jadhao & Pinelli, 2001; Magliulo-Cepriano et al., 1993; Prasada Rao et al., 1996). Additionally, Gal prohormone mRNA is found in the POA-AH of the African cichlid *Astatotilapia burtoni* (Hu et al., 2016). Other hypothalamic Gal-ir neuron populations are also commonly found across teleosts. Though the specific nuclei they are located in vary, Gal-ir somata are commonly reported in the ventral hypothalamus including the ventral tuberal nucleus and the dorsal

zone of the periventricular hypothalamus as well as the caudal hypothalamus. Extrahypothalamic Gal-ir or mRNA-expressing neuron populations have also been detected in several species including the supracommissural nucleus of the ventral telencephalon (Jadhao & Pinelli, 2001; Prasada Rao et al., 1996), periventricular nucleus of the posterior tuberculum (Hu et al., 2016), locus coeruleus (Batten et al., 1990), facial lobe (Jadhao & Pinelli, 2001), and in (Batten et al., 1990) or near (Hu et al., 2016; Prasada Rao et al., 1996) the vagal lobe (midshipman have dense Gal-ir fibers in this region; XL, Figure 4c).

Patterns of telencephalic Gal-ir fiber expression appear to be relatively well conserved across teleosts, which would now include midshipman. Regardless of the region Gal-ir neurons are located, they project broadly throughout the teleost brain. In general, most show dense fibers present in the subpallium/area ventralis of the telencephalon, but sparse to moderate expression only in the pallium/area dorsalis of the telencephalon (Amano et al., 2009; Batten et al., 1990; Cornbrooks & Parsons, 1991; Holmqvist & Ekstrom, 1991; Jadhao & Pinelli, 2001; Olivereau & Olivereau, 1991; Yamamoto et al., 1992). Though sparse in midshipman, labeled fibers are also commonly reported in the olfactory bulb (Amano et al., 2009; Batten et al., 1990; Cornbrooks & Parsons, 1991; Prasada Rao et al., 1996). In the dienkephalon, fibers are reported in the same POA-hypothalamic regions that Gal-ir somata are located: POA, ventral tuberal nucleus, and the dorsal zone of the periventricular hypothalamus. Fibers are also reported in the pituitary for salmonids (*S. salar* (Holmqvist & Ekstrom, 1991; Olivereau & Olivereau, 1991), *S. farior*, *S. gairdneri*, *Oncorhynchus tshawytscha* (Olivereau & Olivereau, 1991), *O. mykiss* (Anglade et al., 1994)); sailfin mollies (Cornbrooks & Parsons, 1991); eels (*Anguilla anguilla*, *A. rostrata*, and *A. japonica*) (Olivereau & Olivereau, 1991); weakly electric fish (*Apteronotus leptorhynchus*) (Yamamoto et al., 1992); "four-eyed" fish (*Anableps anableps*) (Jadhao & Pinelli, 2001); and goldfish (Prasada Rao et al., 1996).

In the brainstem, some regions commonly show expression of Gal-ir fibers, including in midshipman. For the midbrain these include the tectum (Amano et al., 2009; Anglade et al., 1994; Batten et al., 1990; Cornbrooks & Parsons, 1991; Olivereau & Olivereau, 1991; Prasada Rao et al., 1996; Yamamoto et al., 1992), torus semicircularis (Batten et al., 1990; Cornbrooks & Parsons, 1991; Olivereau & Olivereau, 1991; Yamamoto et al., 1992), and less frequently in dorsal tegmentum (Amano et al., 2009; Anglade et al., 1994), at a similar anatomical location as the midshipman PAG. The only mesencephalic region with reported Gal-ir label that is not present in midshipman is found in nucleus tegmenti dorsalis lateralis of Atlantic salmon (Holmqvist & Ekstrom, 1991). Hindbrain distribution of Gal-ir fibers has been described in fewer species compared to forebrain and midbrain, but expression is generally reported in the reticular formation (Anglade et al., 1994; Holmqvist & Ekstrom, 1991), ventral hindbrain (Olivereau & Olivereau, 1991), and more specifically the medulla oblongata (Anglade et al., 1994; Prasada Rao et al., 1996; Yamamoto et al., 1992).

Similar to midshipman, sex differences are also reported in some other teleost species. In most cases of sexual dimorphism, including midshipman, Gal-ir somata populations and fiber distributions are reduced in females compared to males. In sailfin mollies, females have

fewer fibers in tectum, and throughout midbrain, hindbrain, and spinal cord (Cornbrooks & Parsons, 1991). Goldfish males have increased fiber densities in olfactory bulb, dorsal telencephalon, POA, ventral tuberal nucleus, medulla oblongata, and facial lobe, as well as higher numbers of somata in TP and Gal-ir cell populations in PPa and parvocellular division of PM that are absent in females (Prasada Rao et al., 1996). One exception to this trend is the red salmon for which females have a population of Gal-ir somata in PM that is absent in males (Jadhao & Meyer, 2000). However, this overall trend is suggestive for a role for Gal in male-specific behavior in teleosts.

One factor to consider when making comparisons between Gal-ir distribution in midshipman and other teleosts is that while we used a custom antibody raised in guinea pig, prior studies in teleosts have all used commercially available antibodies, mostly raised against porcine Gal (Amano et al., 2009; Anglade et al., 1994; Batten et al., 1990; Holmqvist & Ekstrom, 1991; Jadhao & Meyer, 2000; Olivereau & Olivereau, 1991; Prasada Rao et al., 1996). Because there is some variability between teleost Gal peptide sequences, and significant variation between teleost and porcine Gal (Table 2), it is possible that some of the differences seen between midshipman and other teleosts are due to variation in the specificity of the antibodies used to detect Gal. Nevertheless, Gal-ir distribution in midshipman appears to fit within the range of interspecific variation already reported.

4.4 | Comparisons with tetrapods

Like in teleosts, Gal-ir somata are found in the POA and hypothalamus of amphibians (Lázár et al., 1991; Olivereau & Olivereau, 1992), turtles (Jiménez et al., 1994), birds (Azumaya & Tsutsui, 1996; Jozsa & Mess, 1993; Klein et al., 2006), and mammals (Gentleman et al., 1989; Kordower, Le, & Mufson, 1992; Skofitsch & Jacobowitz, 1985). However, unlike in teleosts, Gal-ir neurons are more commonly found in several extra-hypothalamic regions in tetrapods. This includes multiple forebrain and brainstem sites in frogs (see Moore & Lowry, 1998), cortical expression in rats and primates (Kordower et al., 1992; Skofitsch & Jacobowitz, 1985), amygdala (Jiménez et al., 1994; Lázár et al., 1991; Olivereau & Olivereau, 1992; Skofitsch & Jacobowitz, 1985), septum (Jozsa & Mess, 1993; Kordower et al., 1992; Skofitsch & Jacobowitz, 1985), hippocampus (Jozsa & Mess, 1993; Kordower et al., 1992), and nucleus of the solitary tract (Jozsa & Mess, 1993; Lázár et al., 1991; Olivereau & Olivereau, 1992), which contains the largest extrahypothalamic population of Gal-ir somata in chickens. Overall, distribution of Gal-expressing neurons appears to be expanded in tetrapods compared to teleosts.

Gal-ir fibers are also widely distributed throughout tetrapod brains, as is the case for midshipman and other teleosts. In particular, several of the proposed homologous neuroendocrine, vocal and auditory regions share Gal-ir fiber input between midshipman and tetrapods. This includes regions in the POA and hypothalamus (Azumaya & Tsutsui, 1996; Gentleman et al., 1989; Jiménez et al., 1994; Jozsa & Mess, 1993; Olivereau & Olivereau, 1992; Skofitsch & Jacobowitz, 1985), as well as vocal-motor control centers such as the PAG (Jiménez et al., 1994; Kordower & Mufson, 1990; Lázár et al., 1991; Sandi & Haller, 2015) and the homologous nucleus intercollicularis in

Japanese quail (*Coturnix japonica*) (Azumaya & Tsutsui, 1996; Kingsbury, Kelly, Schrock, & Goodson, 2011). Stimulation of the PAG results in species-specific vocal output (Jürgens, 1994, 2002), including in midshipman (Feng & Bass, 2014; Kittelberger et al., 2006). Gal-ir label is also found in the principal midbrain auditory region, the torus semicircularis, of several other nonmammalian vertebrate species including somata and fibers in frogs (Lázár et al., 1991) and fibers in salamanders (Olivereau & Olivereau, 1992) and turtles (Jiménez et al., 1994). In rats, both fibers and sparse somata are labeled in the inferior colliculus (Skofitsch & Jacobowitz, 1985), which is considered to be homologous to the torus semicircularis of other vertebrates (McCormick, 1992).

Finally, several other regions with proposed homologues in the teleost brain show Gal-ir fibers in tetrapods. These include hippocampus (teleost lateral zone of area dorsalis of the telencephalon) in chicken (Jozsa & Mess, 1993), rat (Skofitsch & Jacobowitz, 1985), monkey (Kordower et al., 1992), and human (Gentleman et al., 1989), bed nucleus of the stria terminalis and amygdala (teleost supracommissural, intermediate, and postcommissural nuclei of area ventralis of the telencephalon) in primates (Gentleman et al., 1989; Kordower et al., 1992) and similar regions of amphibians (Olivereau & Olivereau, 1992) and turtles (Jiménez et al., 1994), and septum (teleost ventral nucleus of area ventralis) across vertebrates (Azumaya & Tsutsui, 1996; Gentleman et al., 1989; Jiménez et al., 1994; Jozsa & Mess, 1993; Klein et al., 2006; Olivereau & Olivereau, 1992; Skofitsch & Jacobowitz, 1985). Finally, the substantia nigra has Gal-ir cell bodies in rats (Skofitsch & Jacobowitz, 1985) and fibers in monkeys (Kordower et al., 1992). The mammalian substantia nigra has been proposed as a partial homologue of the posterior tuberculum of teleosts (Rink & Wullimann, 2001; Wullimann, 2011, 2014).

In addition to immunohistochemical studies of Gal peptide expression in tetrapods, Kohl et al. (2018) recently used viral tracing to map the synaptic targets of Gal-expressing cells in the medial POA of mice. These included several hypothalamic nuclei, including the supraoptic and paraventricular nuclei, which are proposed partial homologues of teleost parvocellular and magnocellular POA-AH, respectively (Forlano & Cone, 2007; Godwin & Thompson, 2012; Herget et al., 2014). Outside of the hypothalamus, they identify synapses in lateral septum, bed nucleus of the stria terminalis, medial amygdala, substantia nigra, ventral tegmental area (also proposed partial homologue of the posterior tuberculum (Rink & Wullimann, 2001; Wullimann, 2011, 2014)), and PAG, among others. While we are unable to conclusively identify the origins of fibers described here, this study in mice provides some guidance in hypothesizing which fibers likely project from preoptic cells versus those projecting from hypothalamic populations.

4.5 | Functional role of galanin

Midshipman Gal neuroanatomy, along with that of other teleosts, is consistent with a role in governing broad behavioral states. The relatively small number of Gal-ir somata we observe in a limited number of hypothalamic nuclei, combined with the extensive fiber distributions present throughout the brain, including in neuroendocrine, sensory, and motor nuclei, suggest that these neurons may be acting to modulate

activity widely to coordinate behaviors. This anatomical data is consistent with our recent transcriptomic work, which found that Gal transcripts are upregulated in nest-holding type I males engaged in courtship (Tripp et al., 2018). Further, we have recently demonstrated that Gal neurons increase their activity during spawning, but not other related social behaviors, specifically in courting type I males (Tripp, Salas-Allende, Makowski, & Bass, 2019). Taken together, these studies are consistent with a role for Gal in governing reproductive tactics; however, it remains unclear what other behavioral role Gal may be playing in midshipman.

Another possible role for Gal neurons is promotion and regulation of parental care behaviors, as seen for Gal neurons in the medial POA of mice (Wu et al., 2014). Ablation of these neurons results in deficits in several behaviors involved in the care of pups. Additionally, Kohl et al. (2018) report that subpopulations of these neurons have unique projection targets, which each in turn regulates separate aspects of care behaviors. They propose that medial POA Gal neurons integrate diverse inputs and organize discrete behavioral outputs that each contribute to parental care. While we did not find activation of POA-AH Gal neurons in midshipman during care for eggs, it remains possible that they play a role in later stages of parental care (Tripp et al., 2019).

These prior studies also provide context for the sexual dimorphism we describe here. While Gal neurons are important for both male and female parental care in mice (Kohl et al., 2018; Wu et al., 2014), female midshipman do not provide care for developing offspring and depart the nest after spawning is completed (Brantley & Bass, 1994); the reduction in Gal systems in females reflects, in part, the absence of parental care behavior. While type II males also lack parental care behavior, they may yet show nest fidelity during the breeding season like type I males (Brantley & Bass, 1994). It may also be the case that the development of Gal neural circuits occurs before morph differentiation in midshipman males. Lastly, gene expression during spawning suggests a role for Gal in behaviors specific to the type I male courting reproductive tactic (Tripp et al., 2018), and Gal neurons in the POA-AH are activated during spawning in courting type I males but not in type II males or females (Tripp et al., 2019). Thus, the fewer and more nuanced differences in Gal neuroanatomy between the two male morphs may reflect Gal function among males being more strongly driven by levels of gene expression and neuron activation rather than by robust, visually obvious differences in neuroanatomy.

4.6 | Concluding comments

In summary, we find that Gal is mainly expressed in cells in the POA-AH in midshipman fish. These cells project broadly throughout the brain including neuroendocrine regions and nuclei involved in audition and vocal-motor control. Taken together, we also observe what would be considered minor differences in Gal-ir distribution between male morphs and major differences between males and females, which have fewer Gal-expressing cells in the POA-AH and far less dense and even absent Gal-ir fiber expression in several midbrain and hindbrain nuclei compared to males. These sex differences indicate that Gal may be playing an important behavioral role for males specifically.

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CONFLICT OF INTEREST

The authors declare no potential conflict of interest.

DATA AVAILABILITY STATEMENT

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

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