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FoxP2 Expression in a Highly Vocal Teleost Fish with Comparisons to Tetrapods

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Abstract
Motivated by studies of speech deficits in humans, several studies over the past two decades have investigated the potential role of a forkhead domain transcription factor, FoxP2, in the central control of acoustic signaling/vocalization among vertebrates. Comparative neuroanatomical studies that mainly include mammalian and avian species have mapped the distribution of FoxP2 expression in multiple brain regions that imply a greater functional significance beyond vocalization that might be shared broadly across vertebrate lineages. To date, reports for teleost fish have been limited in number and scope to nonvocal species. Here, we map the neuroanatomical distribution of *FoxP2* mRNA expression in a highly vocal teleost, the plainfin midshipman (*Porichthys notatus*). We report an extensive overlap between *FoxP2* expression and vocal, auditory, and steroid-signaling systems with robust expression at multiple sites in the telencephalon, the preoptic area, the diencephalon, and the midbrain. Label was far more restricted in the hindbrain though robust in one region of the reticular formation. A comparison with other teleosts and tetrapods suggests an evolutionarily conserved FoxP2 phenotype important to vocal-acoustic and, more broadly, sensorimotor function among vertebrates.

Introduction

The neural topology of vocal communication in humans is complex and to suggest that a single gene is responsible for language as we know it would seem to be overly simplistic. However, a putative "language gene" – *FOXP2* – that encodes the forkhead box protein P2 was reported in 1990 as an autosomal dominant trait causing a severe and specific speech mutation. Inspired by these findings, disrupted FoxP2 phenotypes have since been described in diverse taxa including mammals (house mouse, *Mus musculus*), birds (zebra finch, *Taeniopygia guttata*), and insects (fruit fly, *Drosophila melanogaster*) (human and nonhuman homologs of this gene are upper- and lowercase, respectively). Findings range from highly precise but incorrect spectral characteristics of learned vocalization [e.g., zebra finch: Haesler et al., 2007] and

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Abbreviations used in this paper

ac	anterior commissure
AR	androgen receptors
ARO	estrogen synthetase/aromatase
AT	anterior tuberal nucleus
C	cerebellum
Cc	cerebellar crest
Cg	granule cell layer of the cerebellum
Cm	molecular layer of the cerebellum
CM	corpus mammilare
Cp	central posterior nucleus of the thalamus
D	area dorsalis of the telencephalon
Dc	central zone of the area dorsalis of the telencephalon
DIL	nucleus diffusus of the inferior lobe
Dl	lateral zone of the area dorsalis of the telencephalon
Dm	medial zone of the area dorsalis of the telencephalon
E	entopeduncular nucleus
Eg	eminentia granularis
ER	estrogen receptors
G	nucleus glomerulosus
Had	dorsal division of the habenula
Hav	ventral division of the habenula
Hd	dorsal zone of the periventricular hypothalamus
Hv	ventral zone of the periventricular hypothalamus
III	third ventricle
IL	inferior lobe of the hypothalamus
IP	isthmal paraventricular nucleus
IS	isthmal nucleus
IV	fourth ventricle
LL	lateral lemniscus
LR	lateral recess of the hypothalamus
MLF	medial longitudinal fasciculus
MV	midbrain ventricle
nLL	nucleus of the lateral lemniscus
NT	nervus terminalis ganglion
PAG	periaqueductal gray
PC	Purkinje cells
PG	preglomerular nucleus
PGl	lateral preglomerular nucleus
PGm	medial preglomerular nucleus
PM	magnocellular preoptic nucleus
PMg	gigantocellular division of the magnocellular preoptic nucleus
PPa	anterior parvocellular preoptic nucleus
PPp	posterior parvocellular preoptic nucleus
PPv	periventricular pretectal nucleus
RF	reticular formation
RFl	lateral reticular formation
RFm	medial reticular formation
RT	rostral tegmental nucleus
SV	saccus vasculosus
TeM	mesencephalic tectum
TP	posterior tuberal nucleus
TPp	periventricular nucleus of the posterior tuberculum
TS	torus semicircularis
TSd	deep cell layer of TS
TSp	periventricular cell layer of TS
V	area ventralis of the telencephalon
Vc	central nucleus of V
Vd	dorsal nucleus of V
Vi	intermediate nucleus of V
Vse	trigeminal sensory nucleus
VM	ventromedial nucleus of the thalamus
VMN	vocal motor nucleus
Vp	postcommissural nucleus of V
VPG	vocal pattern generator
VPN	vocal pacemaker neurons
VPP	vocal prepacemaker nucleus
Vs	supracommissural nucleus of V
Vse	trigeminal sensory nucleus
vT	ventral tuberal hypothalamus
Vv	ventral nucleus of V

conflicting reports of effects on the frequency content of unlearned vocalizations (mouse: Shu et al. [2005] and Gaub et al. [2010] found a significant effect in 6- to 10-day-old pups and no effect in 4-day-old pups, respectively) to disrupted tone avoidance and motor learning [mouse: Groszer et al., 2008; Kurt et al., 2012], general motor deficits in locomotor and flight activity [flies: Lawton et al., 2014], and generally a reduced adult weight [mouse: Shu et al., 2005].

Several studies have identified brain regions that express the *FoxP2* gene, a subset of which have been implicated as important substrates for vocalization in primates, rodents, and songbirds [e.g., Takahashi et al., 2008; Campbell et al., 2009; Mendoza et al., 2015]. Studies of teleost fish have been limited to nonvocal/nonsonic species of medaka (*Orizyas latipes*), zebrafish (*Danio rerio*), and weakly electric fish (*Apteronotus leptorhynchus*) [Shah et al., 2006; Itakura et al., 2008; Harvey-Girard et al., 2012]. The current study maps *FoxP2* mRNA expression in the brain of the highly vocal plainfin midshipman (*Porichthys notatus*) that has been the subject of comprehensive mapping of the central vocal, auditory, and neuroendocrine systems that modulate vocal-acoustic behaviors [Fig. 1; see reviews in Bass, 2014; Forlano et al., 2015; Feng and Bass, 2017]. Comparison of the brain expression pattern of FoxP2 in midshipman with findings in other teleosts, as well as in mice and birds, suggests a pattern of evolutionarily conserved FoxP2-expressing brain regions. We also report extensive colocalization of *FoxP2* with steroid-signaling pathways, consistent with a recent study of mice showing androgen-dependent changes in *FoxP2* mRNA expression levels [Bowers et al., 2014].

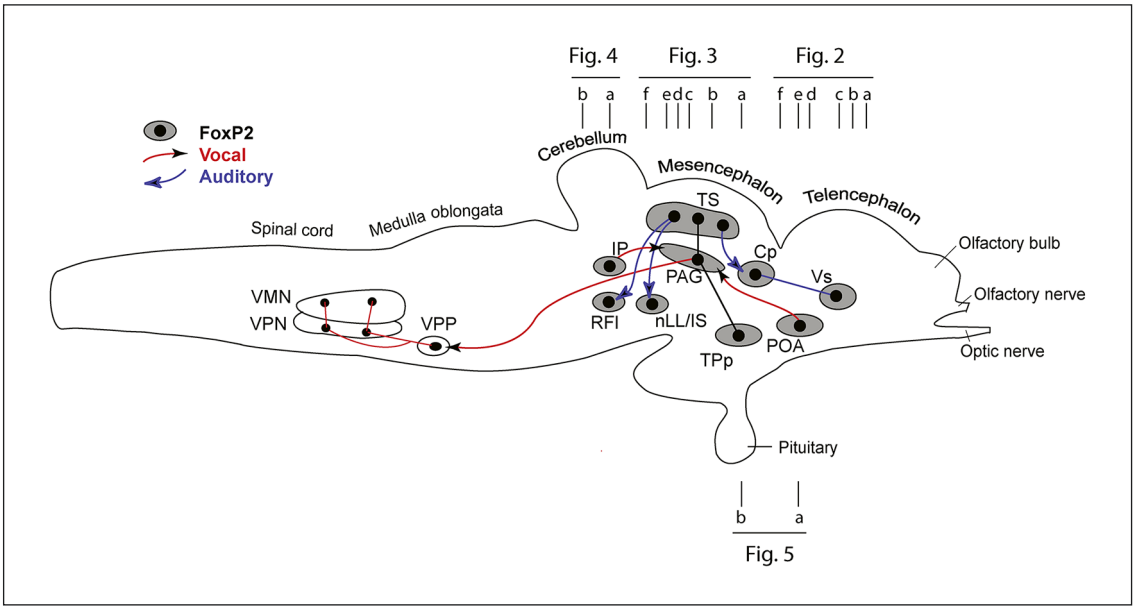

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Fig. 1. Line drawing on the sagittal plane of the brain of a plainfin midshipman fish, *Porichthys notatus*, showing the relative position of major sites of *FoxP2* mRNA expression (dark shading) within major nodes of the vocal and auditory systems [modified from Bass and McKibben, 2003; Kittelberger et al., 2006]. Lines connecting closed circles indicate reciprocal connections and arrowheads indicate unidirectional projection. The approximate levels of the sections illustrated in Figures 2–5 are indicated. Cp, central posterior nucleus of the thalamus; IP, isthmal paraventricular nucleus; IS, isthmal nucleus; nLL, nucleus of the lateral lemniscus; PAG, periaqueductal gray; POA, preoptic area; RFl, lateral reticular formation; TPp, periventricular nucleus of the posterior tuberculum; TS, torus semicircularis; VMN, vocal motor nucleus; VPN, vocal pacemaker nucleus; VPP, vocal prepacemaker nucleus; Vs, supracommissural nucleus of area ventralis.

Materials and Methods

Animals

Midshipman fish were collected from the intertidal zone in northern California (USA) during the breeding season and held in saltwater aquaria for less than 10 days before being sacrificed. Midshipman have two male morphs that follow alternative reproductive tactics and diverge in a suite of anatomical, physiological, and endocrine characters [Bass, 1996; Feng and Bass, 2017]. Type I males build nests and acoustically court females, while type II males sneak or satellite spawn to steal fertilizations from type I males. Here, we did not seek to investigate sex or male morph-specific patterns of *FoxP2* mRNA expression but rather to present a generic map that was based on 1 type I male (13.3 cm in standard length, 27.8 g in mass), 4 type II males (7.9–8.6 cm, 5.3–7.8 g), and 1 female (13.5 cm, 32.9 g). The fish were deeply anesthetized in 0.025% benzocaine and perfused transcardially with a teleost Ringers solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were immediately removed, postfixed in paraformaldehyde-phosphate buffer for 1 h, cryoprotected overnight in 30% sucrose-phosphate buffer, and then frozen at –80 °C in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrence, CA, USA). The frozen brains were later sectioned into 20-µm transverse sections in a –20 °C cryostat and sections were thaw mounted on positively charged microscope slides (Fisher Scientific, Waltham, MA, USA). All procedures were approved by the Institutional Animal Care and Use Committee of Cornell University.

Partial Sequencing of Midshipman FoxP2

A partial sequence of midshipman *FoxP2* was identified from an earlier neural transcriptome study of this species [Feng et al., 2015]. This sequence was later confirmed when cloning a midshipman *FoxP2* fragment derived from a PCR product from midshipman brain cDNA into a bacterial vector for riboprobe synthesis (see section below). The 828-bp sequence fragment was translated and aligned against other vertebrate *FoxP2* protein sequences for comparison over the same sequence region including both the zinc finger and leucine finger domains. Midshipman *FoxP2* shows the highest amino acid identity conservation (84%) with medaka (*Oryzias latipes*: NM_001128513), a species which had one of the highest BLAST hit frequencies of midshipman transcripts from our previous neural transcriptome study [Feng et al., 2015]. Amino acid identity conservation with the midshipman *FoxP2* fragment is 78.6% with zebrafish (*D. rerio*: NM_001030082), 78.1% with a gymnotiform electric fish (*A. leptorhynchus*: JF746164), 73.6% with zebra finches (*T. guttata*: AY549148), 74% with mice (*M. musculus*: NM_053242.4), and 73.6% with humans (*Homo sapiens*: NM_014491.3).

In situ Hybridization

The consensus sequence of *P. notatus Foxp2* was used as a template for primer designs (forward: GCCCATCTCGCACCATTCA, reverse: AGACTGGAGGGCAGGTTCTT) which were ordered from IDT Technologies. *FoxP2* was amplified using PCR on whole-brain cDNA and ligated into a pCRII plasmid vector using

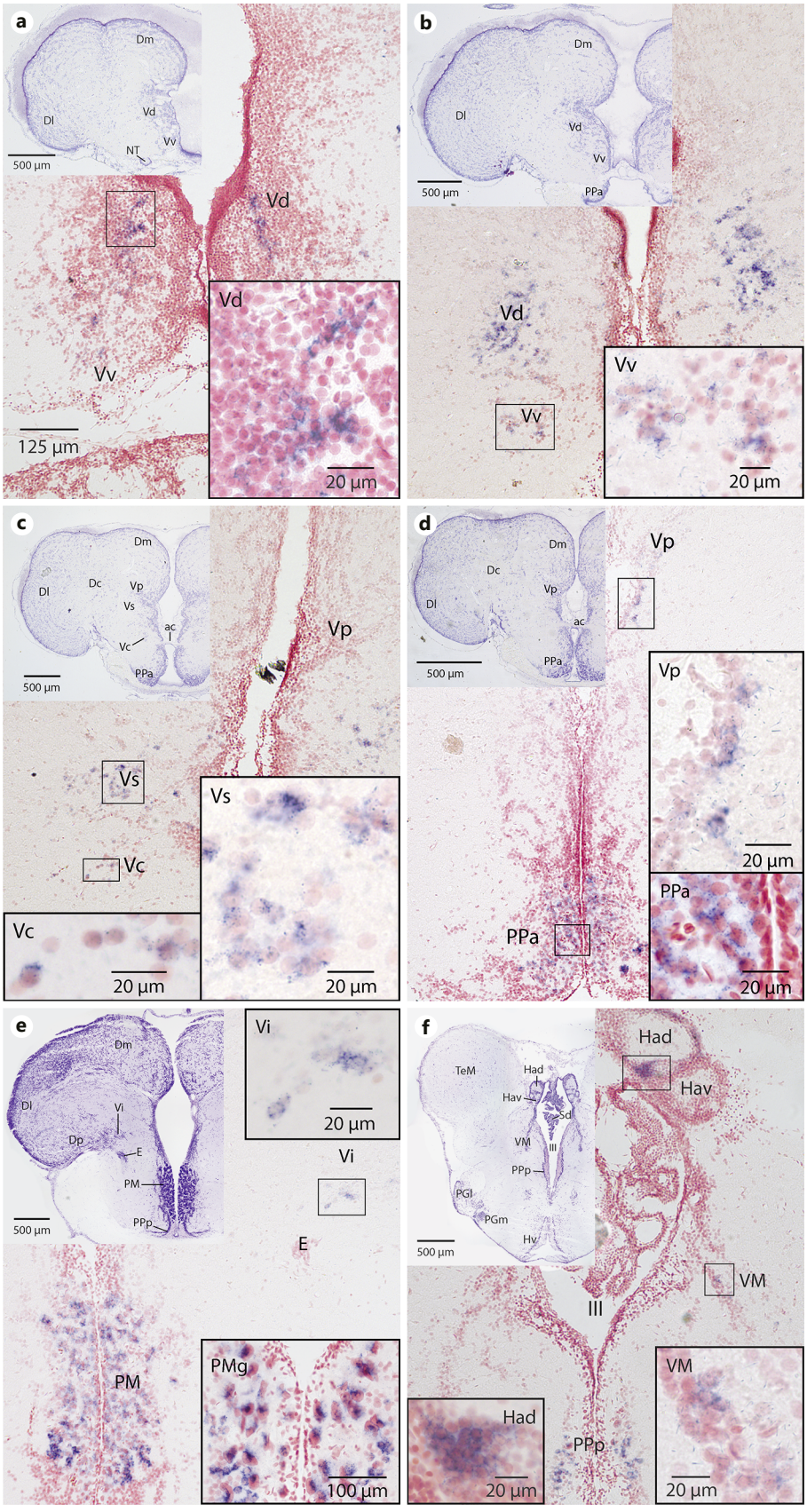

Fig. 2. *FoxP2* mRNA expression in the telencephalon and the preoptic area of a plainfin midshipman fish, *Porichthys notatus*. Each level includes one or two **insets** of photomicrographs superimposed on a neutral red-stained, transverse section showing *FoxP2* mRNA label (blue). Top left **inset** in **a–f** is a low-magnification, cresyl violet-stained transverse section of a comparable level from a reference brain to provide cytoarchitectonic context. Other **insets** are higher-magnification views of the *FoxP2* label from the neutral red-stained section shown, except in **e**, where the PMg **inset** is from a section 180 µm caudal to this level. The area ventralis of the telencephalon exhibits label in dorsal (Vd, **a**), ventral (Vv, **b**), central (Vc, **c**), supracommisural (Vs, **c**), posterior (Vp, **d**), and intermediate (Vi, **e**) nuclei. The preoptic area shows label in anterior parvocellular (PPa, **d**), magnocellular (PM, **e**), gigantocellular (PMg, **e**), and posterior parvocellular (PPp, **f**) divisions. Label is also observed in the dorsal division of the habenula (Had, **f**) and the ventromedial nucleus of the thalamus (VM, **f**). The scale bar in lower left corner of **a** is for each of the low-magnification, neutral red-stained photomicrographs in **a–f**. Each of the **insets** has a separate scale bar. Dm, medial zone of the area dorsalis of the telencephalon; Dl, lateral zone of the area dorsalis of the telencephalon; NT, nervus terminalis ganglion; Dc, central zone of the area dorsalis of the telencephalon; ac, anterior commissure; E, entopeduncular nucleus; Hav, ventral division of habenula; TeM, mesencephalic tectum; PGm, medial preglomerular nucleus; PGl, lateral preglomerular nucleus; Hv, ventral zone of periventricular hypothalamus; III, third ventricle.

a TA Cloning Kit (Life Technologies) and transformed into MAX Efficiency DH5-α™ Competent Cells (Invitrogen) which were cultured and the plasmid vector was extracted by Miniprep (Qiagen). Miniprep samples were submitted for sequencing at Cornell's Genomics Facility and returned 827 bp between designed primers and a 98% match excluding an 83-bp deletion compared to the consensus sequence. Plasmid DNA was then linearized and amplified by PCR using one SP6 promoter primer and one T7 primer to isolate the FoxP2 DNA template containing T7 and SP6 tails. This template was used to synthesize a digoxygenin-labeled cRNA probe using either a T7 or an SP6 RNA synthase enzyme to synthesize the antisense treatment probe or the sense control probe, respectively. RNA was precipitated out of the solution overnight in isopropyl alcohol and lithium chloride at –20 °C and reconstituted in ultrapure water.

Slides were held in RNase-free slide mailers and warmed to room temperature, dried, immersed in 4% PFA, rinsed in DEPC-treated PBS, permeabilized with radioimmunoprecipitation assay buffer, washed in 0.1 M triethanolamine-HCL with 0.25% acetic anhydride, blocked in hybridization buffer at 65 °C, and labeled overnight with digoxygenin-labeled antisense *FoxP2* (treatment) or sense *FoxP2* (control) cRNA at 1 μg/mL in hybridization buffer at 65 °C. Slides were washed in sodium citrate buffer at 62 °C and then blocked in alkaline phosphatase with 0.1% Triton X-100, followed by anti-DIG antibody in 10% horse serum. Finally, the antibody was visualized by incubating in nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT-BCIP; Roche, Indianapolis, IN, USA) until label was visible under a dissection scope. Sections were then counterstained in neutral red, covered in mounting medium, coverslipped, and sealed with VectaMount. All compounds were acquired from Sigma-Aldrich (St. Louis, MO, USA). Sections were viewed on a Nikon Eclipse E800 microscope (Nikon Instruments Inc., Melville, NY, USA) and photographed using an attached digital camera and white balanced using the built-in tool (Spotflex model 15.2, Diagnostic Instruments Inc., Sterling Heights, MI, USA). Photomicrographs were then processed using Photoshop CS4 (Adobe Systems Inc., San Jose, CA, USA) for brightness correction.

Results

We mapped the pattern of *FoxP2* mRNA expression in 14 transverse levels through the brain of a type I male midshipman (13.3 cm in standard length) that were counterstained with neutral red to allow positive identification of brain regions (Fig. 2–4). For each level shown, there is also an inset of a cresyl violet-stained transverse section from reference brain series to show the overall pattern of brain organization at that level. Other insets provide higher-magnification views of label in a subset of brain regions. More detailed cytoarchitecture descriptions of the brain levels illustrated here are available in the studies of Foran et al. [1997], Bass et al. [2000], Goodson and Bass [2002], Forlano et al. [2010], and Kittelberger and Bass [2013]. The nomenclature was adopted from those publications and from Braford and Northcutt [1983].

The qualitative descriptions of the amount of label – robust, moderate, or weak – approximate the apparent density of label in each brain region. With this framework, we describe the amount of label overlapping the area of the corresponding brain region rather than the amount of label per cell. As stated earlier, our overall aim was to establish the presence or absence of *FoxP2* expression in different brain regions to identify a general pattern; hence, no attempt at quantification of *FoxP2* expression was made. There were no qualitative differences in the pattern of label within or between the sexes or morphs, though we realize that a larger sample size (see Materials and Methods) is needed to more conclusively investigate potential sex and morph differences.

FoxP2 mRNA label was concentrated in the neuroplasm. Sections showed virtually no background signal in both unlabeled regions of sections labeled with *FoxP2* antisense mRNA (Fig. 2–4) or in control sections labeled with *FoxP2* sense mRNA (Fig. 5), with the exception of easily identifiable artifacts from unintentionally introduced fibers or dust (e.g., double and single arrows for fibers and dust, respectively, in Fig. 3f and 4a). Four sections now follow describing the pattern of *FoxP2* mRNA label in the telencephalon-preoptic area, the diencephalon, the midbrain-isthmus, and the hindbrain. Only the rostral spinal cord that includes caudal parts of the vocal pacemaker-motoneuron circuit [see Bass et al., 1994] was examined, but there was no identifiable label and so a description of this region is not included.

Telencephalon-Preoptic Area

Antisense *FoxP2* mRNA was expressed in several nuclei of the area ventralis (V), the ventral division of the telencephalon. The furthest rostral label was in the dorsal and ventral nuclei of V (Vd and Vv, respectively; Fig. 2a, b, highlighted in the insets). While the label in Vd was robust throughout its rostral-caudal extent, the label in Vv was relatively weak by comparison. Caudal to Vd, moderate to robust label was found in a lateral, cell-sparse region of the supracommissural nucleus of V (Vs; Fig. 2c, highlighted in the inset) that is a major telencephalic node in the central vocal-acoustic system [Fine and Perini, 1994; Goodson and Bass, 2002]. Ventral to Vs, the central nucleus of V (Vc; Fig. 2c, highlighted in the inset), a small semicircular cluster of somata at the level of the anterior commissure (ac; see cresyl violet inset in Fig. 2c), showed weak label. The posterior nucleus of V (Vp; Fig. 2d, highlighted in the inset) expressed a band of moderate label in

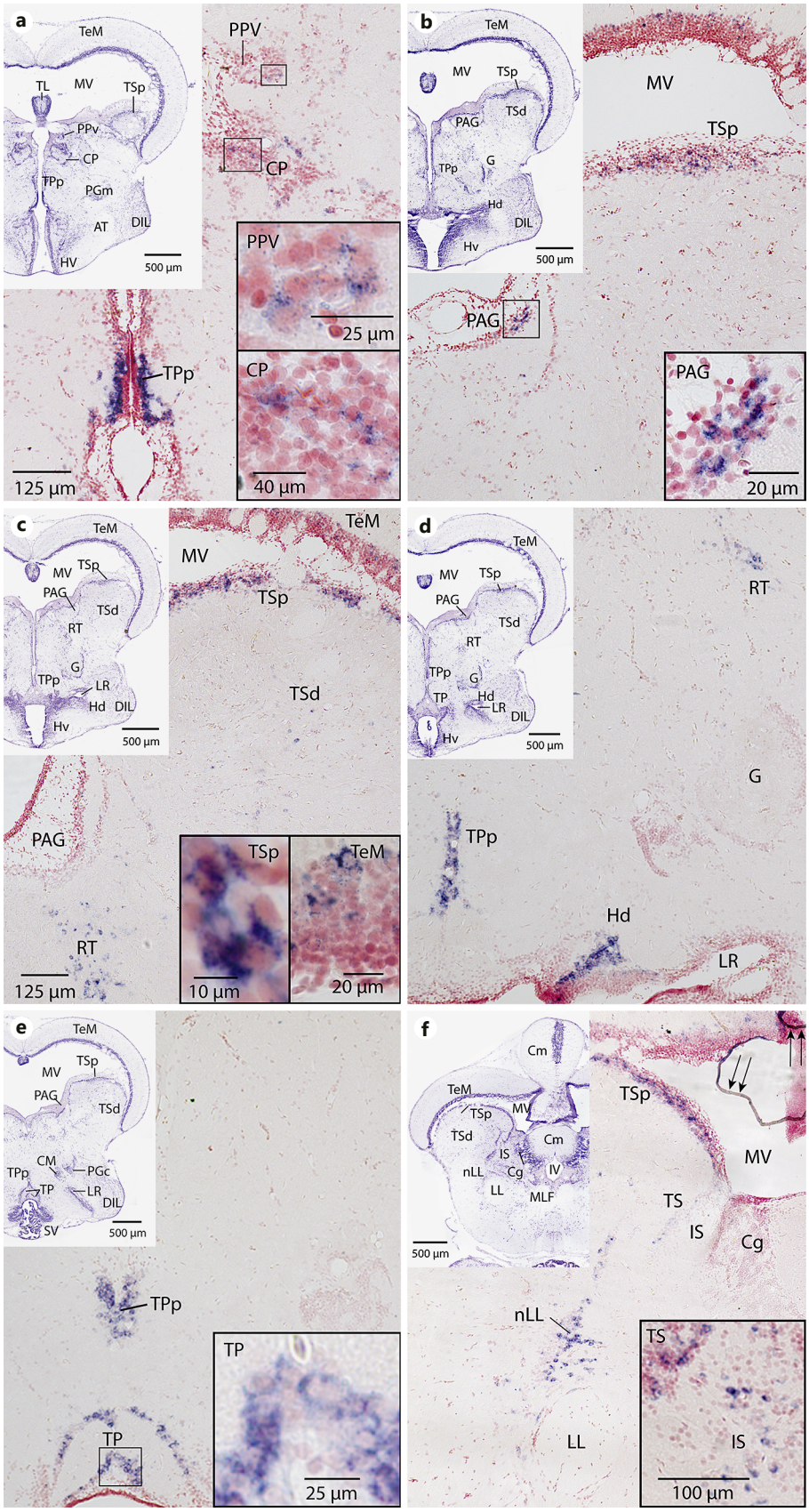

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Fig. 3. *FoxP2* mRNA expression in the diencephalon and midbrain of a plainfin midshipman fish, *Porichthys notatus*. Each level includes one or two **insets** of photomicrographs superimposed on a neutral red-stained, transverse section showing FoxP2 mRNA label (blue). The top left **inset** in **a–f** is a low-magnification, cresyl violet-stained transverse section of a comparable level from a reference brain to provide cytoarchitectonic context. **Insets** in each section are a higher-magnification view of *FoxP2* label (blue) of the same neutral red-stained section, except in **c**, where the TSp **inset** is from a section 80 µm caudal to this level (the TeM **inset** is from the same level but out of view and so lacks a boxed area in the lower power photomicrograph), and in **f** where the IS **inset** is from a section 160 µm caudal to this level. Diencephalic nuclei with *FoxP2* include the ventral part of the periventricular pretectal nucleus (PPv, **a**), the periventricular nucleus of the posterior tuberculum (TPp, **d–e**), the central posterior nucleus of the thalamus (CP, **a**), the posterior tuberal nucleus (TP, **e**), and the dorsal zone of the periventricular hypothalamus (Hd, **d**). Midbrain nuclei with label include the periventricular and deep layers of the torus semicircularis (TSp, TSd, **b**, **c**), the periaqueductal gray (PAG, **b**), the tectum mesencephali (TeM, **c**), rostral tegmental nucleus (RT, **c**), the nucleus of the lateral lemniscus (nLL, **f**), and the isthmal nucleus (IS, **f**). The double arrows in **f** indicate artifacts. The scale bar in the lower left corner of **a** is for each of the low-magnification, neutral red-stained photomicrographs in **a–f**. Each of the **insets** has a separate scale bar. MV, midbrain ventricle; PGm, medial preglomerular nucleus; AT, anterior tuberal nucleus; DIL, nucleus diffusus of the inferior lobe; Hv, ventral zone of the periventricular hypothalamus; G, nucleus glomerulosus; LR, lateral recess of the hypothalamus; CM, corpus mammilare; Cm, molecular layer of cerebellum; SV, saccus vasculosus; Cg, granule cell layer of the cerebellum; IV, fourth ventricle; LL, lateral lemniscus; MLF, medial longitudinal fasciculus; TS, torus semicircularis.

its caudal pole along the lateral aspect of a narrow plate of somata. There was moderate label along the medial aspect of the intermediate nucleus of the area ventralis (Vi; Fig. 2e, highlighted in the inset).

The preoptic area showed an especially robust pattern of *FoxP2* mRNA expression. Like Vs, the preoptic area is a major forebrain vocal-acoustic node [Fine and Perini, 1994; Goodson and Bass, 2002]. Rostrally, this included a dense cluster of somata along the ventral midline of the anterior parvocellular preoptic nucleus (PPa; Fig. 2d, highlighted in the inset). Caudal and ventral to PPa, the magnocellular nucleus of the preoptic area (PM; Fig. 2e) showed especially robust *FoxP2* expression throughout its extent. This included the gigantocellular division of PM at the caudal end of PM that has the largest somata in the preoptic area (PMg; inset in Fig. 2e, 160 μm caudal to the rest of the PM illustrated in Fig. 2e). The posterior parvocellular division (PPp; Fig. 2f) exhibited robust *FoxP2* expression over a dense vertical band of somata that were adjacent to a dense, unlabeled midline cell layer that is likely comprised of glial cells [see Forlano et al., 2001, for the distribution of glial cells].

Diencephalon

There was *Foxp2* mRNA label in several diencephalic nuclei. The most rostral such nucleus was the habenula where robust label appeared along an outer cell-dense region of a dorsal division (Had; Fig. 2f, highlighted in the inset along the border with the ventral division, Hav). At this same level, moderate *FoxP2* expression occurred in the ventromedial nucleus of the thalamus (VM; Fig. 2f, highlighted in the inset). Moderate levels of expression were found further caudal in the central posterior nucleus (CP; Fig. 3a, highlighted in the inset), the dorsal thalamic target of the torus semicircularis (TS) that is the main auditory recipient nucleus in the midbrain [Bass et al., 2000]. Dorsal to CP, there was weak label in the nucleus pretectalis periventricularis, pars ventralis (PPv; Fig. 3a, highlighted in the inset), a multimodal nucleus that projects to the cerebellum and receives input from the retina, the midbrain tectum (TeM), the lateral line division of the TS, and the trigeminal sensory nucleus [see Xue et al., 2007].

In the more ventral posterior tuberculum, the periventricular nucleus of the posterior tuberculum (TPp; Fig. 3a) that is lateral to the third ventricle at the level of the posterior commissure showed the most robust *FoxP2* mRNA label in the brain, overlapping a dense layer of somata. Like CP, TPp is a major node in the central auditory system, in this case providing a prominent dopaminergic input to the peripheral and central auditory systems [Forlano et al., 2014; Perelmuter and Forlano, 2017]. Label was also especially robust throughout the entire extent of a similarly dense cell layer of the posterior tuberal nucleus (TP; Fig. 3d, e) that sits ventral to TPp at more caudal levels and splits into two adjoining ellipsoid-shaped cell clusters along the ventral midline (Fig. 3e, highlighted in the inset).

The dorsal zone of the perventricular hypothalamus (Hd; Fig. 3d; intermediate nucleus of the dorsal periventricular hypothalamus of Rink and Wullimann [2001]) was the only hypothalamic region that expressed *FoxP2*. It showed robust label in a cell-dense region extending dorsolaterally to a ventral layer of somata along the lateral recess (LR, Fig. 3f) that had, by comparison, relatively weak label.

Midbrain

FoxP2 mRNA expression in the midbrain was found along the entire ventricular layer of the midbrain ventricle (MV; Fig. 3). There was robust *FoxP2* expression throughout the periventricular layer of the auditory division of the TS and a deep layer of the TS (TSp, TSd; e.g., Fig. 3b, c, highlighted in the inset in Fig. 3c). The vast majority of somata in the midbrain tectum of midshipman (TeM) are densely packed in the periventricular layer adjacent to the midbrain ventricle [Brantley and Bass, 1988], coincident with moderate *FoxP2* label that is spread over its entire rostral-to-caudal extent (Fig. 3c, highlighted in the inset).

The periaqueductal gray (PAG) forms a crescent of somata immediately adjacent to the auditory TS and is a major site of integration in the vocal-auditory system of midshipman fish [see Kittelberger and Bass, 2013]. Robust *FoxP2* expression was confined to somata of the lateral part of the rostral PAG (Fig. 3b, highlighted in the inset). Robust label also occurred over a cluster of somata in the rostral tegmental nucleus (RT; Fig. 3c) just ventral to the caudal aspect of the PAG.

At caudal levels of the TS, robust label occurred over the nucleus of the lateral lemniscus (nLL) that sits immediately dorsal to the lateral lemniscus (Fig. 3f) and receives a dense input from the auditory TS [Bass et al., 2000].

Hindbrain Including the Isthmus Region

At caudal midbrain levels, the isthmal nucleus (IS; Fig. 3f, highlighted in the inset taken 160 μm caudal) had robust levels of *FoxP2* expression at its caudal end that became weak at its rostral end. This nucleus receives in-

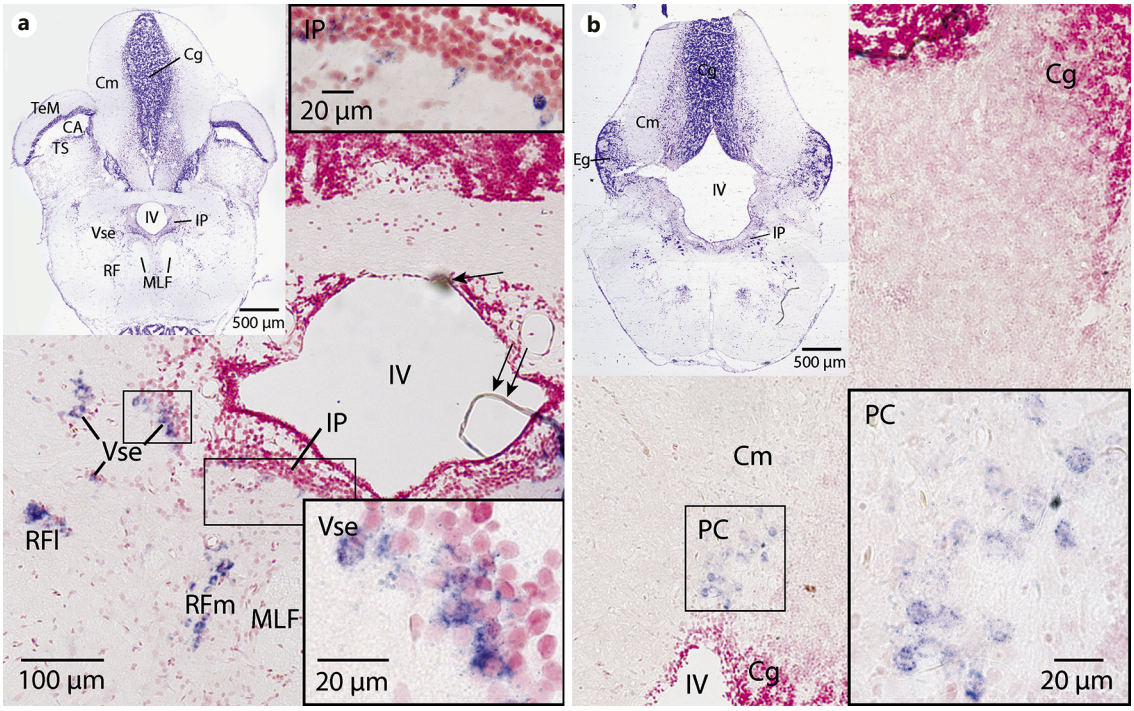


Fig. 4. *FoxP2* mRNA expression in the hindbrain of a plainfin midshipman fish, *Porichthys notatus*. Each level includes one or two **insets** of photomicrographs superimposed on a neutral red-stained, transverse section showing *FoxP2* mRNA label (blue). The top left **inset** in **a** and **b** is a low-magnification, cresyl violet-stained transverse section of a comparable level from a reference brain to provide cytoarchitectonic context. Hindbrain regions with *FoxP2* mRNA expression label include the isthmal paraventricular nucleus (IP, **a**), medial and lateral dvisions of the reticular formation (RFm, RFl, **a**), the sensory nucleus of the trigeminal (Vse, **a**), and Purkinje cells in the caudal lobe of the cerebellum (PC, **b**). The double and single arrows in **a** indicate artifacts. The scale bar in lower left corner of **a** is for both of the low-magnification, neutral red-stained photomicrographs. Each of the **insets** has a separate scale bar. Cg, granule cell layer of the cerebellum; Cm, molecular layer of cerebellum; IV, fourth ventricle; MLF, medial longitudinal fasciculus; RF, reticular formation; RFL, lateral reticular formation; TeM, mesencephalic tectum; TS, torus semicircularis.

put from the auditory TS and vocal central pattern generator (CPG) in the hindbrain, has reciprocal connections with the PAG, and should not be confused with the nucleus isthmi that receives TeM inputs [see Bass et al., 1994, 2000; Kittelberger and Bass, 2013]. Weak *FoxP2* label was observed in the isthmal paraventricular nucleus (IP; Fig. 4a, highlighted in the inset) that extends from the level of nLL to mid-levels of the cerebellum, is connected to the vocal CPG, and projects to the PAG [Bass et al., 1994; Goodson and Bass, 2002; Kittelberger and Bass, 2013].

The medial reticular formation (RFm; Fig. 4a) expressed robust label over a nearly vertical band lateral to the medial longitudinal fasciculus (MLF; Fig. 4a). Lateral and slightly dorsal to RFm, there was robust label over a separate cluster of cells (RFl; Fig. 4a). Dorsal to RFl and RFm, and lateral to IP, the trigeminal sensory nucleus expressed a moderate amount of label in three separate clusters of somata (Vse; Fig. 4a, highlighted in the inset). Lastly, Purkinje cell somata (PC; Fig. 4b, highlighted in the inset) in the caudal lobe of the cerebellum were the most caudal hindbrain site showing *FoxP2* label. These cells were sparsely distributed within the molecular layer of the caudal lobe (Cm; Fig. 4b) adjacent to the granule cell layer (Cg; Fig. 4b) along the fourth ventricle [see Bass, 1982, for cytoarchitecture of the cerebellum, and Straka et al., 2006, for a description of caudal lobe PC].

Discussion

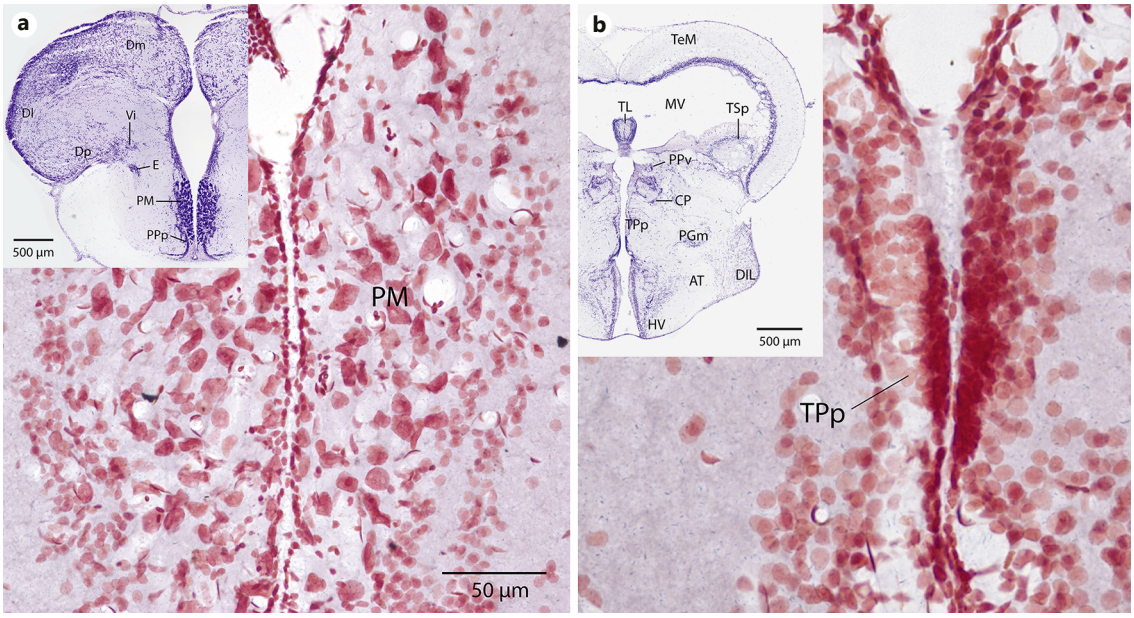
Midshipman fish have been extensively studied as a model system for the neural and hormonal mechanisms underlying vocal-acoustic communication [e.g., Bass and Remage-Healey, 2008; Forlano et al., 2014; Bass et al., 2015; Feng and Bass, 2017]. Here, we report robust *FoxP2*

Table 1. Neuroanatomical distribution in the midshipman brain of FoxP2 mRNA and steroid hormone-signaling pathways

Anatomical location	FoxP2	AR	ARO	ERα	ERβ1	ERβ2
Telencephalon						
Ventral nucleus of the area ventralis (Vv) – auditory	+	–	+	+	–	+
Dorsal nucleus of the area ventralis (Vd)	+	+	+	–	–	+
Supracommissural nucleus of the area ventralis (Vs) – auditory, vocal	+	+	+	+	–	+
Postcommissural nucleus of the area ventralis (Vp) – auditory	+	+	+	+	–	–
Central nucleus of the area ventralis (Vc)	+	–	–	–	–	–
Intermediate nucleus of the area ventralis (Vi)	+	+	+	–	–	–
Preoptic area						
Anterior parvocellular preoptic nucleus (PPa) – auditory, vocal	+	+	+	+	+	+
Posterior parvocellular preoptic nucleus (PPp) – auditory, vocal	+	+	+	–	–	+
Magnocellular preoptic nucleus (PM/PMg)	+	+	+	+	+	–
Diencephalon						
Habenula (Had)	+	–	–	–	–	–
Ventromedial nucleus of the thalamus (VM)	+	–	–	–	–	–
Central posterior nucleus (CP) – auditory	+	+	+	+	–	–
Periventricular pretectal nucleus (PPv)	+	–	–	–	–	–
Periventricular nucleus of the posterior tuberculum (TPp) – auditory	+	+	+	+	–	+
Posterior tuberal nucleus (TP)	+	+	+	–	–	+
Dorsal zone, periventricular hypothalamus (Hd)	+	+	+	+	–	+
Midbrain-isthmal region						
Periacqueductal gray (PAG) – vocal	+	+	+	–	+	+
Mesencephalic tectum (TeM)	+	+	–	+	–	–
Periventricular cell layer of the torus semicircularis (TSp) – auditory	+	+	–	–	–	+
Deep cell layer of the torus semicircularis (TSd)	+	–	–	–	–	–
Nucleus of the lateral lemniscus (nLL) – auditory, vocal	+	–	–	–	–	–
Rostral tegmental nucleus (RT)	+	–	+	–	+	–
Isthmal nucleus (IS) – vocal	+	–	–	–	–	–
Isthmal pariventricular nucleus (IP) – vocal	+	–	–	–	–	–
Hindbrain						
Reticular formation (RFm, RFl)	+	+	+	–	–	–
Trigeminal sensory nucleus (VSe)	+	–	–	–	–	–
Purkinje cells (PC)	+	–	–	–	–	–

This table is modified from Forlano et al. [2016]. Cell groups that are major nodes in the central auditory and/or vocal network are indicated. AR, androgen receptors; ARO, estrogen synthetase/aromatase; ER, estrogen receptors.

mRNA label in the forebrain and midbrain, with more limited expression in the hindbrain (Table 1). The most robust label was found in TPp of the diencephalon, a nucleus that has been compared to the ventral tegmental area/substantia nigra and the A11 dopaminergic population in the diencephalon [Wullimann, 2009, 2011; Filipi et al., 2014]. Label was also robust in two ventral telencephalic nuclei, i.e., Vd and Vs, that have been compared, in part, to basal ganglia (Vd) and subpallial amygdala (Vs) of tetrapods [Mueller et al., 2008; Maximino et al., 2013], as well as in the preoptic area. Though label was not found in the neuronal populations comprising the hindbrain vocal CPG (VMN, VPP, and VPN) [Bass and Baker, 1990; Chagnaud et al., 2011, 2012; Chagnaud and Bass, 2014], robust or moderate label was identified in forebrain and midbrain sites that are major nodes in the central vocal and auditory networks (Fig. 1; Table 1). Forebrain regions include TPp, Vs, and the preoptic area. Midbrain sites include the PAG that has direct input to the vocal CPG and receives direct input from three prominent FoxP2 mRNA sites: the preoptic area; the periventricular and deep layers of the TS (TSp and TSd) that have reciprocal connections with the PAG; and the isthmal region, IS, that also has reciprocal connections with the PAG [Bass et al.,



Color version available online

Fig. 5. Transverse sections showing the absence of sense *FoxP2* mRNA label in two brain regions with robust antisense *FoxP2* mRNA label, the magnocellular division of the preoptic area (PM, **a**), and the periventricular nucleus of the posterior tuberculum (TPp, **b**). Compare **a** with Figure 2e and b with Figure 3a. The top left **inset** in **a** and **b** is a low-magnification, cresyl violet-stained transverse section of a comprable level from a reference brain to provide cytoarchitectonic context. The scale bar in **a** is for each of the low-magnification, neutral red-stained photomicrographs in **a** and **b**. AT, anterior tuberal nucleus; DIL, nucleus diffusus of inferior lobe; Dl, lateral zone of the area dorsalis of the telencephalon; E, entopeduncular nucleus; MV, midbrain ventricle; PGm, medial preglomerular nucleus; PPp, posterior parvocellular preoptic nucleus; PPv, periventricular pretectal nucleus; TeM, mesencephalic tectum; TSp, periventricular cell layer of the torus semicircularis; Vi, intermediate nucleus of the area ventralis of the telencephalon.

2000; Goodson and Bass, 2002; Bass et al., 2005; Kittelberger and Bass, 2013]. Auditory nuclei with robust or moderate *FoxP2* label that are targets of auditory TS efferents were nLL in the midbrain and CP in the dorsal thalamus [Bass et al., 2000]. Recent evidence points to TPp, which had the most prominent *FoxP2* label, as a major site providing modulatory input to multiple nodes in the vocal-auditory network. Dopaminergic neurons in TPp innervate the PAG, the auditory division of the inner ear, the saccule, and a rostral hindbrain nucleus (octavolateralis efferent nucleus; OEN) that also innervates the saccule [Forlano et al., 2014; Perelmuter and Forlano, 2017] and is the source of a vocal corollary discharge that relays information such as call duration from the vocal CPG circuit [Chagnaud and Bass, 2013].

The report of Bowers et al. [2014] that androgens influence *FoxP2* mRNA and/or protein levels in the striatum, the cerebellar vermis, and the cortex of mice during embryonic and postnatal development led us to compare sites of *FoxP2* expression in midshipman with steroid-signaling pathways (Table 1). Two thirds of the *FoxP2*-positive sites express androgen (AR) and/or estrogen (ERα, ERβ1, and ERβ2) receptors or the enzyme aromatase (estrogen synthase) that converts testosterone to estrogen [Table 1; see Forlano and Bass, 2011, for review]. Together with studies in avian species showing the expression of steroid-signaling pathways in the auditory and song control systems [e.g., Ball et al., 2004; Caras and Remage-Healey, 2016], these findings suggest a more phylogenetically widespread functional relationship between steroid-signaling pathways and FoxP2 that is worthy of further investigation.

Comparisons with Other Teleosts

With this report, FoxP2 expression has now been reported in the brain of four species within the superorder Acanthopterygia – zebrafish and gymnotids (Cypriniformes) that are more closely related and basal within the teleost lineage than medaka (Ovalentariae) and midshipman (Batrachoidiformes) [Betancur et al., 2013]. Prominent *FoxP2* mRNA label in zebrafish is reported in general for the ventral telencephalon and the preoptic area,

with more specific sites identified in the diencephalon (dorsal thalamus, ventral posterior tuberculum), the midbrain (periventricular pretectum, TeM, TS), the medulla (superior reticular nucleus, medial octavolateralis nucleus), and the caudal lobe of the cerebellum [Shah et al., 2006; see Bonkowsky and Chien, 2005, for studies during embryogenesis]. For midshipman, we recognize label in several divisions of the ventral telencephalon and the preoptic area and in cerebellar Purkinje cells of the caudal lobe (Table 1). The dorsal thalamic, posterior tuberculum, and pretectal regions identified in zebrafish likely correspond to CP, TPp, and PPv, respectively, in midshipman, and the zebrafish superior reticular nucleus corresponds to either RFl or RFm in midshipman (Table 1). The medial octavolateralis nucleus, a lateral line recipient nucleus, had no apparent label in midshipman.

The report for weakly electric gymnotiform fish focuses on the telencephalon and the preoptic area [Harvey-Girard et al., 2012]. As in midshipman, *FoxP2* mRNA label is reported in several divisions of the ventral telencephalon – Vv, Vd, and Vc – and in the PPa [Harvey-Girard et al., 2012]. Gymnotiforms mainly differ from midshipman and other species in showing *FoxP2* label in the central, lateral, dorsal, and medial divisions of the area dorsalis of the telencephalon.

Sites identified with a FoxP2 antibody in medaka include the PPa, the thalamus (VM), the periventricular hypothalamus (Hd), TeM, and Purkinje cells [Itakura et al., 2008]. Label is also reported for the nervus terminalis ganglion, the epiphysis, and the medial division of the area dorsalis (DM), regions that did not show label in either midshipman or zebrafish and with the exception of DM are also not reported in gymnotiform fish. The retina was also labeled in medaka, a tissue that others and we did not investigate.

Taken together, midshipman, zebrafish, medaka, and gymnotiforms share patterns of FoxP2-positive sites in the ventral telencephalon, the preoptic area, the diencephalon (thalamus, posterior tuberculum, pretectum, and hypothalamus), the midbrain tectum (TeM) and torus (TS), and the cerebellum (Purkinje cells). One major exception in midshipman is the absence of label in the area dorsalis of the telencephalon that is reported for medaka and gymnotiforms. Of particular significance to the current study of a highly vocal teleost is that midshipman mainly differ from these other species in having robust *FoxP2* label in a telencephalic site, Vs, and several midbrain-isthmal sites – PAG, nLL, and IS – that are major nodes of the central vocal-acoustic network (Fig. 1) [Bass et al., 1994, 2000; Goodson and Bass, 2002; Kittelberger and Bass, 2013]. Though not unique to midshipman, the robust *FoxP2* label in TPp is worthy of note in this context given its prominent input to the OEN and hence an implied role in the vocal corollary discharge pathway [Chagnaud and Bass, 2013].

Comparisons with Birds and Mammals

We compared FoxP2 sites in midshipman brain with those in birds and mammals for brain regions where homologous comparisons have been proposed. We consider the midshipman pattern to also be largely representative of the teleost species so far studied given the general overlap in expression patterns (see above). At the outset, it is important to note that both birds and mammals show expression of FoxP2 in many regions in which teleost fish apparently do not express it, at least based on our current understanding of homologous cell groups between these lineages, and we therefore excluded these regions from our comparisons.

A comparison of FoxP2 expression among four species of mice – *Scotinomys teguina*, *S. xerampelinus*, *Peromyscus maniculatus*, and *M. musculus* – shows a highly conserved pattern with few interspecific differences [Campbell et al., 2009]. A conserved pattern of FoxP2 expression is also observed between avian vocal learners and nonlearners [Haesler et al., 2007; Chen et al., 2013; Mendoza et al., 2015; also see Reiner et al., 2004, for current avian nomenclature]. In light of these similarities within each taxon, we consider the available results reported for *M. musculus* and zebra finch as representative of mice and birds, respectively. For comparisons to zebra finch, we specifically combine data from Mendoza et al. [2015], Chen et al. [2013], and Vicario et al. [2017] into the analysis. Homologous patterns of gene expression are considered to be any case where midshipman, mouse, and zebra finch each express FoxP2 in a comparable region in at least one study. The reader is referred to Forlano and Bass [2011] for a summary comparing forebrain regions in midshipman to those in tetrapods, but also see Wullimann [2009, 2011], O'Connell and Hofmann [2011], Maximino et al. [2013], and Biechl et al. [2017] for teleosts in general; also see Mueller et al. [2008] for comparisons to the striatum and the subpallium. For the midbrain PAG, see Goodson and Bass [2002] and Kittelberger and Bass [2013] for midshipman, and Kingsbury et al. [2011] for birds. For more specific teleost comparisons of dorsal telencephalic sites, see Harvey-Girard et al. [2012] who reported the most extensive label for those regions in gymnotids.

Table 2. Expression patterns of FoxP2 across diverse vertebrate lineages

Midshipman	Mouse	Zebra finch
Telencephalon		
Supracommissural, intermediate and posterior nuclei of the area ventralis (Vs, Vi, Vp)	Extended amygdala, bed nucleus of the stria terminalis	Extended amygdala, bed nucleus of the stria terminalis
Dorsal and central nuclei of the area ventralis (Vd, Vc)	Striatum	Striatum (including Area X)
Ventral nucleus of the area ventralis (Vv)	Septum	?
Preoptic area (POA)		
Anterior parvocellular nucleus (PPa)	POA (parvocellular nuclei)	?
Posterior parvocellular nucleus (PPp)		
Magnocellular division of magnocellular nucleus (PM)		
Gigantocellular division of the magnocellular preoptic nucleus (PMg)	Paraventricular nucleus	
Diencephalon		
Central posterior nucleus (CP)	Medial geniculate	Nucleus ovoidalis
Habenula	Habenula	Habenula
Posterior tuberculum (TPp, TP)	Substantia nigra/ventral tegmental area or A11[1]	Substantia nigra/ventral tegmental area[1]
Midbrain		
Tectum mesencephali (TeM)	Superior colliculus	Optic tectum
Torus semicircularis (TS)	Inferior colliculus	MLd (nucleus mesencephalicus lateralis, pars dorsalis)
Nucleus of the lateral lemniscus (nLL)	Dorsal nucleus of the lateral lemniscus	Nucleus lemnisci lateralis
Periaqueductal gray (PAG)	Periaqueductal gray	Nucleus intercollicularis
Hindbrain		
Purkinje cells of the cerebellum	Purkinje cells of the cerebellum	Purkinje cells of the cerebellum
Reticular formation	Reticular formation	Reticular formation

[1] Comparisons of the posterior tuberculum to the substantia nigra/ventral tegmental area or A11 population of diencephalic dopaminergic neurons remain unresolved – see Discussion. Question marks indicate that it is unclear if this has been investigated.

FoxP2-positive sites shared between midshipman fish, mice, and birds (Table 2) include the extended amygdala/bed nucleus of the stria terminalis, the striatum, dorsal thalamic auditory nuclei (CP, medial geniculate, and nucleus ovoidalis), habenula, midbrain visual and auditory nuclei (tectum/superior colliculus, torus semicircularis/inferior colliculus/MLd), nuclei associated with the lateral lemniscus (nLL/dorsal nucleus of the lateral lemniscus/nucleus lemnisci lateralis), the periaqueductal gray, the ventral tegmental area/substantia nigra (VTA/SN) or A11 dopaminergic neurons, cerebellar Purkinje cells, and hindbrain reticular formation. Additional shared FoxP2-positive sites between midshipman and mice include the septum and preoptic area.

We compare the posterior tuberculum to two sites in tetrapods: the VTA/SN as proposed by Wullimann [2009, 2011] and the A11 population of dopaminergic neurons in the diencephalon [Lindvall et al., 1974; Takada et al., 1988] as proposed by Driever and colleagues [Filippi et al., 2014]. Support for a comparison to the VTA/SN comes from reports of FoxP2 expression in the VTA/SN of birds and mammals [see Haesler et al., 2007, for adult songbirds, and Campbell et al., 2009, for adult mice]; the caveat here is the lack of colocalization of FoxP2 and tyrosine hydroxylase in the VTA/SN of adult mice [Wijchers et al., 2006]. Support for a comparison to A11 comes from developmental studies of dopaminergic cell groups in zebrafish and mammals [see Filippi et al., 2014]; the caveat here is the apparent lack of a report for FoxP2 label in A11. Given that TPp has more than one subdivision [Rink and Wullimann, 2002], it may yet be an amalgam of neurons comparable to both the VTA/SN and the A11 populations. To help resolve these comparisons, it would be important to carry out colocalization studies of FoxP2 and dopamine/tyrosine hydroxylase in midshipman fish or zebrafish.

Shared patterns of robust FoxP2 expression in the auditory and visual systems implies a conserved and salient role for FoxP2 in the integration of these sensory modalities with motor function as reflected in FoxP2 expression

in comparable cell populations of the striatum, substantia nigra/VTA, PAG, cerebellum, and reticular formation. These interpretations strongly resonate with those of Campbell et al. [2009], specifically that the widespread expression of FoxP2 "is unlikely to selectively regulate circuits governing verbal and vocal functions." Furthermore, our findings confirm the evolutionarily ancient nature of FoxP2 as "integrally, although not exclusively, involved in pathways that subserve modulation of fine motor output, multimodal sensory processing, and sensorimotor integration" [Campbell et al., 2009]. Homologous expression of FoxP2 among these taxa in the striatum, substantia nigra-VTA, and PAG further suggests that such highly conserved elements could mediate some of the nonspecific FoxP2-related impairments to fine motor behavior and that selectively avoiding these areas for FoxP2 knockdown might mitigate some of its nonspecific effects.

The unexpected overlap between FoxP2 expression and steroid-signaling pathways is consistent with the available evidence in mammals for the influence of steroids on FoxP2 expression, and of both steroids and FoxP2 on vocal mechanisms in birds and mammals that merit further investigation among other groups of bony vertebrates, both fish and tetrapods. This could include the observed overlap between fish and mammals in FoxP2 expression in the extended amygdala/BNST and preoptic area, sites that are especially rich in steroid- and other hormone-signaling pathways [e.g., see Goodson and Bass, 2001]. Double label, in situ immunohistochemical experiments in midshipman of aromatase or steroid receptors [e.g., Forlano et al., 2001; Fergus and Bass, 2013] with FoxP2 could help to establish the identity of the FoxP2-expressing cells in these overlapping regions.

In a similar vein, double-labeling studies could offer stronger support for some of the proposed homologies for regions that we report as being FoxP2-positive sites in the brain of midshipman fish. For example, FoxP2-positive catecholaminergic neurons identified by double labeling with tyrosine hydroxylase [e.g., see Forlano et al., 2014] would more clearly resolve comparisons of TPp with the VTA and the SN and the A11 population of dopaminergic neurons. Similarly, cholinergic FoxP2-positive cells identified by double labeling with choline acetyltransferase [e.g., see Brantley and Bass, 1988] would help with forebrain comparisons, as would double-labeling FoxP2 neurons with transcription factors such as pallidal genes [e.g., see Mueller et al., 2008].

As evidenced by this overlap, FoxP2 likely also has unexpected functionalities that should be considered in the context of their contributions to behavioral vocal deficits seen in FoxP2 mutant and knockdown experiments. FoxP2 may wear many functional "hats" depending on the lens through which we choose to view it, and it may serve as an evolutionarily ancient genetic substrate upon which many systems, including vocalization, have evolved dependence rather than considering it primarily a "language gene" that affects other systems as well.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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