Exposure to Advertisement Calls of Reproductive Competitors Activates Vocal-Acoustic and Catecholaminergic Neurons in the Plainfin Midshipman Fish, *Porichthys notatus*

Christopher L. Petersen¹, Miky Timothy¹, D. Spencer Kim¹, Ashwin A. Bhandiwad², Robert A. Mohr², Joseph A. Sisneros²,³, Paul M. Forlano¹,⁴,⁵

¹Department of Biology, Brooklyn College, City University of New York, Brooklyn, New York, United States of America, ²Department of Psychology, University of Washington, Seattle, Washington, United States of America, ³Virginia Bloedel Hearing Research Center, Seattle, Washington, United States of America, ⁴Aquatic Research and Environmental Assessment Center, Brooklyn College, Brooklyn, New York, United States of America, ⁵Programs in Neuroscience, and Ecology, Evolution, and Behavior, The Graduate Center, City University of New York, New York, New York, United States of America

Abstract

While the neural circuitry and physiology of the auditory system is well studied among vertebrates, far less is known about how the auditory system interacts with other neural substrates to mediate behavioral responses to social acoustic signals. One species that has been the subject of intensive neuroethological investigation with regard to the production and perception of social acoustic signals is the plainfin midshipman fish, *Porichthys notatus*, in part because acoustic communication is essential to their reproductive behavior. Nesting male midshipman vocally court females by producing a long duration advertisement call. Females localize males by their advertisement call, spawn and deposit all their eggs in their mate’s nest. As multiple courting males establish nests in close proximity to one another, the perception of another male’s call may modulate individual calling behavior in competition for females. We tested the hypothesis that nesting males exposed to advertisement calls of other males would show elevated neural activity in auditory and vocal-acoustic brain centers as well as differential activation of catecholaminergic neurons compared to males exposed only to ambient noise. Experimental brains were then double labeled by immunofluorescence (-ir) for tyrosine hydroxylase (TH), an enzyme necessary for catecholamine synthesis, and cFos, an immediate-early gene product used as a marker for neural activation. Males exposed to other advertisement calls showed a significantly greater percentage of TH-ir cells colocalized with cFos-ir in the noradrenergic locus coeruleus and the dopaminergic periventricular posterior tuberculum, as well as increased numbers of cFos-ir neurons in several levels of the auditory and vocal-acoustic pathway. Increased activation of catecholaminergic neurons may serve to coordinate appropriate behavioral responses to male competitors. Additionally, these results implicate a role for specific catecholaminergic neuronal groups in auditory-driven social behavior in fishes, consistent with a conserved function in social acoustic behavior across vertebrates.

Introduction

Vocalizations are key components of social behavior in many vertebrates. Perception of these social acoustic signals may elicit communicative responses and/or reproductive behavior in conspecifics and is essential for mating in many teleost fishes [1,2], anuran frogs [3], and songbirds [4]. Various forms of vertebrate sociality including but not limited to consummatory and appetitive sexual behavior and aggression are mediated by a highly conserved and reciprocally connected suite of nuclei within the basal forebrain and midbrain termed the social behavior network (SBN) [5–7]. Catecholaminergic circuitry, including the ascending dopaminergic system, has been suggested to work in concert with the SBN to assess the salience of socially relevant stimuli, and to reinforce appropriate behavioral responses to such social stimuli [7,8]. Interestingly but not surprisingly, key components of the neural circuitry that underlie vocal-acoustic behavior reside in the SBN and are highly conserved throughout vertebrate taxa [6,9]. Although the neural circuitry responsible for encoding acoustic stimuli is well delineated in several vertebrate taxa, very little is known about how the auditory system interacts with the SBN to mediate responses to social acoustic signals. The neural substrates that allow vertebrates to produce vocalizations share similar developmental origins and vocal-acoustic communication is thought to have first evolved in teleost fishes, the most speciose vertebrate group [10]. As such, elucidating the interaction between
the SBN and auditory circuitry in teleosts will provide a better understanding of the function and evolution of these systems in other vertebrates.

One teleost system that has become a powerful neuroethological model for investigating vocal-auditory behavior among vertebrates is the plainfin midshipman fish (*Porichthys notatus*), in part because the production and perception of social acoustic signals is essential to the reproductive success of this species [1,11–13]. The plainfin midshipman is a marine teleost fish in the family Batrachoididae that migrates seasonally (spring/summer) from deep offshore sites to spawn in rocky intertidal zones off of the northwest coast of the United States [11]. Type I or “nesting” males produce a long duration (>1 min) vocalization or “hum” that serves as an advertisement call to attract females. Females find courting males by localizing the hum, spawn once by depositing eggs on the roof of the type I male’s nest, and then return to deep offshore sites [14]. After fertilization, type I males guard their nest and continue to vocally court females for additional spawnings throughout the breeding season. As multiple type I males establish nests in close proximity to one another, an acoustic environment with concurrent overlapping hums is created whereby resident nesting males may be able to access and respond acoustically to the hums of neighbors [1]. The perception of potential competitors may affect an individual’s motivational state and subsequently elicit appropriate social responses.

In teleost fishes, the encoding of auditory stimuli first occurs in the saccule [15–17], the main endorgan of hearing in midshipman and most other teleosts, which projects via the eighth nerve to the descending octaval (DO) nucleus in the auditory hindbrain [18–20]. The rostral intermediate division of DO (DOri) is not only a major source of ascending afferent projections to the midbrain torus semicircularis (TS), but also projects to the preprecemaker nucleus of the vocal pattern generator [9,18,19] and thus provides a vocal-auditory interface at the level of the auditory hindbrain in the medulla. Nucleus centralis within TS (TSnc) is largely medial and periventricular, and has been physiologically identified as an auditory center which can encode concurrent hums [21,22] and shares reciprocal connections with the central posterior nucleus (CP, auditory thalamus) [9,19] as well as with the anterior tuberal hypothalamus (AT). Finally, CP has reciprocal connections with AT and the ventral tuberal hypothalamus (vT), both of which are part of the descending vocal-motor circuitry and SBN [6,9].

In the closely related Gulf toadfish, *Opsanus beta* (same family as the midshipman fish), exposure to conspecific advertisement calls is known to increase vocal production and raise circulating blood levels of 11-ketotestosterone and cortisol [23]. While acoustic playback challenges are known to elicit simultaneous changes in circulating hormone levels, vocal behavior and territoriality [24], the physiological response to such a challenge may not be limited to changes in steroid levels alone. Catecholamines (CA), which are important social and sexual behavior in other species [34–37]. However, the function of these CA groups and whether or not they are important modulators of social behavior in teleost fishes is unknown. In midshipman, like other teleosts, connectivity of DAergic TPp neurons and TPp in general [38] appear to make them ideal candidates for sensorimotor (including auditory-vocal) integration and higher order decision making [32,39].

The goal of this study was to characterize the role of a vocal fish responsive to advertisement calls of potential competitors. To this end we presented wild-caught type I midshipman males (*P. notatus*) with playback of advertisement calls of field-recorded midshipman, and examined patterns of neural activity by assaying changes in cFos, an immediate early gene product, within the CNS. Specifically, we tested the hypothesis that type I male midshipman exposed to other advertisement calls would show elevated neural activity (i.e., increased cFos response) in auditory and vocal-auditory brain centers as well as differential activation of CA neurons compared to control males exposed only to ambient noise. We quantified cFos immunoreactive (ir) neurons in major auditory nuclei including DOri, TSnc, and CP, as well as in two vocal-auditory centers (AT, vT) which are both part of the teleost SBN. We also examined whether the social acoustic signals increased the cFos response in CA neuronal populations in the LC and TPp by double labeling with tyrosine hydroxylase (TH), the rate-limiting enzyme in CA synthesis. Our results are the first to demonstrate a link between the exposure to social acoustic signals and the activation of specific catecholaminergic nuclei along with brain regions associated with auditory processing and social behavior in a teleost fish. In addition, our data support the hypothesis that CAs are important neurochemicals involved in the modulation of auditory-driven social behavior across vertebrates.

**Brain Activation to Social Acoustic Signals**

**Ethics Statement**

All experimental animal procedures performed in this study were approved by the Institute for Animal Care and Use Committee of the University of California, Davis (Protocol Number: 15977), and animals were collected from the field under California Department of Fish and Game Permit 802021-01.

**Animals**

Male plainfin midshipman fish (*Porichthys notatus*) were collected from nests during the morning low tides at several natural breeding locations in Tomales Bay near Marshall, CA, USA, in the same geographical locations used in previous studies over the last 20 years (e.g., [40–42]). Type I males were distinguished from type II’s [11] and females by size, and sex was confirmed post-sacrifice. Type I males were transferred to coolers with aerated sea water and then transported to the UC Bodega Marine Laboratory (BML) in Bodega Bay, CA where they were housed in flow-through sea water aquaria until play-back trials within 24–72 hrs of collection. Holding time between collection and playback should not have affected the fishes’ sensory capabilities as previous studies showed no decrease in female midshipman auditory sensitivity to encoding frequencies of the male mate call until more than 25 days post-collection [16]. At BML, the fish were maintained in large communal tanks at natural ambient temperatures (12–14°C) until playback experiments were conducted.

**Playback Experiment**

All tests were conducted at BML in an outdoor, cylindrical concrete tank (4 m diameter, 0.75 m depth) at night between
eliminated specific fluorescent signal. Specificity for both the TH [45,46] and cFos [44,47] antibodies which time they were sealed with nail polish and stored at 4°C overnight.

ProLong Gold with DAPI (Life Technologies), and allowed to dry.

Slides were then washed 4 X 10 min in PBS, and coverslipped with ProLong Gold with DAPI (Life Technologies), and allowed to dry in the dark at room temperature for approximately 48 hours at which time they were sealed with nail polish and stored at 4°C. Specificity for both the TH [45,46] and cFos [44,47] antibodies have been demonstrated elsewhere in teleost fishes. Additionally, controls without primary or secondary antibodies or preabsorption of cFos primary antibody with its blocking peptide (SC-253P, Santa Cruz Biotechnology) as per manufacturer’s instruction, eliminated specific fluorescent signal.

Immunohistochemistry

Slides were allowed to warm to room temperature and were washed 3 times for 15 minutes in phosphate buffered saline (PBS; pH 7.2) followed by 1 hour soak in 0.3% Triton X-100 in PBS (PBST) +10% blocking solution [8% normal donkey serum (DS, Jackson Immunolab, West Grove, PA) and 0.5% normal donkey serum (PBS-0.5%)] and incubated for 16 hours at room temperature in PBST-0.83% blocking solution [9% normal donkey serum (DS, Jackson Immunolab, West Grove, PA)+2% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) (PBST-DS/BSA)]. After blocking, tissue was incubated for 16 hours at room temperature in PBST-DS/BSA containing mouse anti-tyrosine hydroxylase (TH, 1:1000 Millipore, Temecula, CA) and rabbit anti-cFos (1:2000 Santa Cruz Biotechnology, Santa Cruz, CA). Post incubation, slides were washed 5 X 10 min in PBS +0.5% normal donkey serum (PBS-DS). This was followed by 2 hour incubation in PBST-DS/BSA+donkey anti-mouse conjugated to Alexa Fluor 488 (dilution 1:400, Life Technologies, Norwalk, CT), and donkey anti-rabbit conjugated to Alexa Fluor 568 (1:200, Life Technologies). Slides were then washed 4 X 10 min in PBS, and coverslipped with ProLong Gold with DAPI (Life Technologies), and allowed to dry in the dark at room temperature for approximately 48 hours at which time they were sealed with nail polish and stored at 4°C. Specificity for both the TH [45,46] and cFos [44,47] antibodies have been demonstrated elsewhere in teleost fishes. Additionally, controls without primary or secondary antibodies or preabsorption of cFos primary antibody with its blocking peptide (SC-253P, Santa Cruz Biotechnology) as per manufacturer’s instruction, eliminated specific fluorescent signal.

Image Acquisition and Anatomy

Auditory/Vocal-Acoustic centers. Images were acquired on an Olympus BX61 epifluorescence compound microscope (Tokyo, Japan) using MetaMorph imaging and processing software (Molecular Devices, Sunnyvale, CA). Each nucleus analyzed in the auditory/vocal-acoustic system was identified at low magnification and photomicrographed with a 20x objective at the same light level and exposure time. Each photomicrograph was taken consecutively using Texas Red and DAPI filter sets (Chroma, Bellows Falls, VT) within a z-stack at 5 levels (2 levels above and 2 levels below a central plane), each 1 μM thickness. These 5 photomicrographs were combined into a single projected image in MetaMorph. For each image, cFos-ir signal was thresholded above background [48], and the number of cFos-ir cells was quantified using MetaMorph’s integrated morphometry analysis (IMA) feature. A size filter in IMA was employed to count only immunofluorescence ≥125 pixels. This value was determined a priori as the average pixel count for the smallest size of cFos-ir cells we considered to be signal. cFos-ir cells that were clumped were counted manually: a region was created around the cFos-ir cell(s) in question, and transferred to the DAPI channel where the presence of multiple nuclei was confirmed. Sampling strategy was determined per region to account for intrinsic variation in size between nuclei [49]. In the case of tissue loss or damage, the opposite side of the brain was used (for unilateral sampling), or the section was omitted (see below). In each animal, the average number of cFos-ir neurons per section was calculated per nucleus. Experimenter was blind to treatment conditions of all slides analyzed.

The most basal level of the ascending auditory pathway we analyzed was the rostral intermediate division of the descending octaval nucleus (DOri). Beginning rostrally in DOri, (Figure 1A) we sampled serial sections caudally until its disappearance at the level of the octavolateralis effrent nucleus (OE) (Fig. 1B) [18,19]. A border was drawn around DOri in the DAPI channel and transferred to the Texas red channel to quantify the number of cFos-ir neurons. Landmarks from published descriptions of this nucleus were used to identify its extent [19]. As DOri is directly innervated by nVIII [19], we quantified cFos-ir neurons bilaterally to account for any activation bias that may have occurred due to the position of the fish relative to the speaker. On average, 10.8 sections were analyzed (±3.7 SD). An independent samples t test was performed to determine that there was no difference in number of sections used between groups (p>0.83).

In the auditory midbrain, we quantified numbers of cFos-ir neurons within an area drawn around the periventricular Tsnc (Figure 1C–D) in 2 adjacent photomicrographs captured with 20x objective at each level sampled. Medially, this area excluded the periaqueductal gray (PAG) and paratoral tegmentum (PTT) which are both known to elicit vocal behaviors in physiological preparations [9]. Laterally, landmarks were used to assure that there was no overlap in photomicrographs. We began analysis of Tsnc at the level of the molecular cell layer of the valvula (Vm). Moving in the caudal to rostral direction, we sampled every fourth section unilaterally until the appearance of the auditory thalamus (CP). The right side of the brain was used unless there was damage to the tissue, in which case we used the left side. Five sections throughout Tsnc were used to calculate average cFos-ir neurons per section, except for 1 animal in the social signal group where 4 sections were used. Teral efferens projects to both the compact (CPr) and diffuse (CPd) divisions of the central posterior nucleus (CP) of the thalamus (Figure 1E) [19]. CPr forms a wing-shaped band of cells lateral to the midline, and CPd is a loose collection of cells which extend ventrolaterally from CPr [9]. A boundary was...
Figure 1. Auditory and vocal-acoustic anatomy. Transverse sections with DAPI nuclear counterstain (blue) showing white borders around nuclei in which numbers of cFos-ir neurons were quantified within auditory and vocal-acoustic pathways. The caudal (A) and rostral (B) extents of the rostral intermediate descending octaval nucleus (DOiri). The caudal (C) and rostral (D) extents of the periventricular nucleus centralis of the torus semicircularis (TSnc). (E) Compact (CPc) and diffuse (CPd) divisions of the central posterior nucleus. Ventromedial to CP is the anterior tuberal nucleus (AT) in the ventral hypothalamus. (F) Ventral tuberal nucleus (vT) of the anterior hypothalamus. (G) (inset) Dorsal view of midshipman brain with relative positions of A–F. Abbreviations: Cerebellum (C); Cerebral Aqueduct (CA); Horizontal commissure (HoC); Internal arcuate fiber tract (iaf);
Brain Activation of Ascending Auditory Nuclei

Morphological analyses were performed on all fish used in the study. For the social signal exposed males, standard length (SL) = 16.4 ± 0.8 cm (mean ± SD), body mass (BM) = 74.5 ± 10.2 g, and gonadosomatic index (GSI) = 0.62 ± 0.31. For the ambient noise exposed males, SL = 17.0 ± 1.3 cm, BM = 72.7 ± 15.7 g, and GSI of 0.33 ± 0.34. There were no differences in the body metrics (SL, BM, and GSI) between social signal and ambient noise exposed males (p > 0.4 for all cases).

Brain Activation of Ascending Auditory Nuclei

Numbers of cFos-ir neurons were analyzed between groups in the hindbrain DoRi (Fig. 3A–B), the midbrain TSnc (Fig. 3C–D), and 2 subdivisions of CP (CPc and CPd) (CPc shown in Fig. 3E–F) within the auditory thalamus. Males exposed to social acoustic
signals had a significantly greater number of cFos-ir neurons in DOr (t(10) = 3.19, p = 0.01, Fig. 3G) and TSc (t(10) = 2.89, p < 0.05, Fig. 3H) than males exposed to ambient environmental noise. We found that social signal males also had significantly greater numbers of cFos-ir neurons in CPc (t(10) = 4.14, p < 0.01), as well as CPd (t(10) = 4.68, p = 0.001). Values for both divisions of CP were combined in each section throughout the nucleus as a total measure of neural activity in the auditory thalamus. Social signal males were found to have more cFos-ir neurons in the auditory thalamus than ambient noise controls (CP (t(10) = 4.56, p = 0.001, Fig. 3I). Taken together, these data show that males exposed to other male’s advertisement calls show a significantly greater activation at three levels of the ascending auditory system over exposure to ambient environmental noise.

Brain Activation of Hypothalamic Vocal-Acoustic Nuclei

Exposure to social acoustic signals had a significant effect on the numbers of cFos-ir neurons in one of the two SBV/vocal-acoustic nuclei that we analyzed. The hypothalamic AT (Fig. 4A–B) which connects to both the ascending auditory and descending vocal motor pathways showed a greater number of cFos-ir neurons in social signal over control males (t(8) = 3.24, p = 0.01, Fig. 4C). While the average number of cFos-ir neurons was higher in the vT of social signal males (Fig. 4D–E), this difference did not reach statistical significance (t(7) = 2.13, p = 0.07, Fig. 4F).

Activation of CA Neurons

Catecholaminergic neurons within the NAergic LC (Fig. 5D–E) and the DAergic TPp (Fig. 5A–B) were differentially activated (as assayed by TH/cFos-ir colocalization) in male midshipman exposed to social acoustic signals over those exposed to ambient environmental noise. Males exposed to other male advertisement calls had a significantly greater percent colocalization of cFos-ir in TH-ir LC (t(10) = 5.53, p < 0.001, Fig. 5F) and TPp neurons (t(9) = 3.21, p = 0.01, Fig. 5C) compared to controls. Importantly, there was no difference in the total number TH-ir neurons analyzed between groups in either the LC (Social Stimulus: 36.7 ± 6.9 SD, Ambient Noise: 43.0 ± 10.1; p > 0.5) or the TPp (Social Stimulus: 109±82, Ambient Noise: 211±51; p > 0.5).

Co-activation of Auditory/Vocal-Acoustic Nuclei and CA Neurons

Finally, we performed pair-wise correlations to determine if there was a functional relationship between the number of cFos-ir neurons within the auditory/vocal acoustic circuitry with the
Figure 3. cFos response to social acoustic signals (advertisement calls) in the ascending auditory pathway. Representative images of cFos-ir neurons (red/pink) within males who were exposed to social acoustic signals (left column, A, C, E) versus males who were exposed to ambient environmental noise (right column, B, D, F). (A,B) Rostral intermediate division of the descending octaval nucleus (DOri) within the medulla. (C,D) The periventricular nucleus centralis within the midbrain torus semicircularis (TSnc). (E,F) Compact division of the central posterior nucleus (CP) in the
auditory thalamus. Scale bar = 100 μm. Arrows represent the dorsal (D) and lateral (L) orientation for each image. Data in G–I are represented as mean number of cFos-ir neurons per section ± SE, *p<0.05 **p≤0.01, ***p<0.001.

doi:10.1371/journal.pone.0070474.g003

Figure 4. cFos response to social acoustic signals (advertisement calls) in hypothalamic vocal-acoustic circuitry. Representative images of cFos-ir neurons (red/pink) within the anterior tuberal nucleus (AT) (A,B) and ventral tuberal nucleus (vT) (D,E) of males who were exposed to social acoustic signals versus males exposed to ambient environmental noise. Data in C and F are represented as mean number of cFos-ir neurons per section ± SE, **p=0.01. Scale bar = 100 μm. Arrows represent the dorsal (D) and lateral (L) orientation for each image.

doi:10.1371/journal.pone.0070474.g004

Figure 4. cFos response to social acoustic signals (advertisement calls) in hypothalamic vocal-acoustic circuitry. Representative images of cFos-ir neurons (red/pink) within the anterior tuberal nucleus (AT) (A,B) and ventral tuberal nucleus (vT) (D,E) of males who were exposed to social acoustic signals versus males exposed to ambient environmental noise. Data in C and F are represented as mean number of cFos-ir neurons per section ± SE, **p=0.01. Scale bar = 100 μm. Arrows represent the dorsal (D) and lateral (L) orientation for each image.

doi:10.1371/journal.pone.0070474.g004
percentage of TH-ir neurons colocalized with cFos-ir neurons within the dopaminergic periventricular posterior tuberculum (TPp) and the noradrenergic locus coeruleus (LC) of males exposed to social acoustic signals and males exposed to ambient noise. Data in C and F are represented as mean percent colocalization ± SE, **p<0.01, ***p<0.001. Scale bar = 100 μm. Arrows represent the dorsal (D) and lateral (L) orientation for each image.

doi:10.1371/journal.pone.0070474.g005

Figure 5. cFos-ir colocalization with catecholaminergic (TH-ir) neurons. Arrowheads indicate cFos-ir colocalized to catecholaminergic neurons within the dopaminergic periventricular posterior tuberculum (TPp) (A, B) and the noradrenergic locus coeruleus (LC) (D, E) of males exposed to social acoustic signals and males exposed to ambient noise. Data in C and F are represented as mean percent colocalization ± SE, **p<0.01, ***p<0.001. Scale bar = 100 μm. Arrows represent the dorsal (D) and lateral (L) orientation for each image.

doi:10.1371/journal.pone.0070474.g005

percentage of TH-ir neurons colocalized with cFos-ir in the LC and TPp. In the ascending auditory pathway, LC had a significant positive correlation with DOri \( r = 0.63, p<0.05 \) (Fig. 6A), TSnc \( r = 0.73, p<0.01 \) (Fig. 6C) and CP \( r = 0.79, p<0.01 \) (Fig. 6E). TPp had significant positive correlation with CP \( r = 0.70, p<0.05 \) (Fig. 6F). There was no correlation between percent colocalization in TPp with DOri \( r = 0.51, p=0.11 \) (Fig. 6B), or with TSnc \( r = 0.29, p=0.38 \) (Fig. 6D). In the vocal-acoustic pathway, LC had
Brain Activation to Social Acoustic Signals

Locus Coeruleus (Noradrenergic)

- DOri cFos-ir Neurons
  - $r = 0.63$, $p < 0.05$

- ISNe cFos-ir Neurons
  - $r = 0.73$, $p < 0.01$

- CP cFos-ir Neurons
  - $r = 0.79$, $p < 0.01$

- AT cFos-ir Neurons
  - $r = 0.83$, $p < 0.01$

- VT cFos-ir Neurons
  - $r = 0.71$, $p < 0.05$

TPp (Dopaminergic)

- Social Acoustic: Ambient noise
  - $r = 0.51$, $p = 0.11$

% TH-ir Neurons with cFos-ir
Figure 6. Co-activation of auditory/vocal-acoustic nuclei and CA neurons. Pairwise correlations between numbers of cFos-ir neurons in auditory/vocal acoustic nuclei and percent colocalization of cFos-ir within tyrosine hydroxylase (TH-ir) neurons of locus coeruleus (LC) (A–E) and the periventricular posterior tuberculum (TPp) (F–J). Closed circles are males exposed to social signals; open circles are males exposed to ambient noise; green trend lines indicate significant correlations (p ≤ 0.05). doi:10.1371/journal.pone.0070474.g006

a significant positive correlation with both AT (r = 0.83, p < 0.01 Fig. 6G) and vT (r = 0.71, p < 0.05 Fig. 6I), while TPp correlated positively only with AT (r = 0.77, p < 0.05; vT: r = 0.54, p = 0.17 Fig. 6H/J respectively).

Discussion

The localization of immediate early gene transcripts and products has been established as a reliable method for mapping the neural response to a variety of stimuli [55,56] including auditory stimuli in mammals [57], birds [58–60] and anuran frogs [61–64]. Here we demonstrate that immediate early gene products such as cFos can be used to map the neural response to social acoustic signals in teleost fish, the vertebrate group where vocal-acoustic communication is thought to have first evolved [10]. Our results corroborate numerous neurophysiological and neuroanatomical tract-tracing studies that have delineated the auditory [19,21,22], vocal-acoustic [9,18,38,65] and social behavior network [6] circuitry of this species. We show that males exposed to social acoustic signals (advertisement calls of other males) had a greater number of cFos-ir neurons across several levels of the ascending auditory pathway, at least one level of the descending vocal-motor circuit, and in two key catecholaminergic neuronal populations compared to fish exposed to ambient noise. Importantly, these results are the first to show activation within catecholaminergic nuclei after exposure to a social acoustic stimulus in any fish, and, consistent with studies in tetrapods, support the hypothesis that catecholamines are important neuromodulators of vocal-acoustic communication across vertebrates.

cFos-ir Response in Ascending Auditory Circuitry

As predicted, the social acoustic signal-induced significant increases in numbers of cFos-ir cells in auditory processing centers in the hindbrain (DOri), midbrain (TSnc) and thalamus (CP) compared to ambient noise alone. The descending octaval nucleus (DO) is directly innervated by nVIII, projects to midbrain TS and represents a first-order auditory nucleus within the CNS of the midshipman fish [18,19], as well as other teleosts [19,66–69]. Importantly, in midshipman, adaptive reproductive-related changes in frequency encoding occur in the inner ear, implying that social incentive processes begin even at the level of the auditory periphery [13,16,17]. The rostral intermediate subdivision (DOri) that we analyzed in this study also has a direct connection to the prepackemaker nucleus of the vocal pattern generator and thus is designated as part of the hindbrain vocal-acoustic complex that can serve to provide feedback during calling [9,18,19,70].

In anamniote vertebrates (e.g., cartilaginous and bony fishes and anuran frogs), tertiary auditory processing occurs in the torus semicircularis (TS) which is the midbrain homologue to the mammalian inferior colliculus [19,61,71,72]. In the plainspotted midshipman, the periventricular nucleus centralis (nc) has been physiologically identified as the primary auditory center within TS [19], and participates in the ability to discriminate between the physical properties of overlapping advertisement calls [21,22]. Similar to our findings in midshipman, transcripts for cfos as well as another immediate early gene egr-1 were found to be differentially increased in response to socially relevant auditory stimuli in various subdivisions of TS in male Tungara frogs (Physalaemus pustulosus) [61,63]. Unlike the anuran TS which has multiple cytoarchitecturally distinct subdivisions with differing efferent and afferent connections within the auditory circuit [61,71,72], nC is the primary auditory center within TS of the midshipman [19]. The teleost TS does have functionally discrete subregions such as the nucleus ventrolateralis (TSnv) which is the midbrain target of the ascending lateral line system [73]. TSnv is both anatomically and functionally distinct from TSnc, and was excluded from our analysis.

TSnc supplies the primary auditory input into the compact and diffuse divisions of the central posterior nucleus (CPc and CPd) within the thalamus [9,19]. Whether or not CPc and CPd serve discrete roles in auditory processing and integration is unknown. To date, only Lu and Fay [74] have recorded from teleost neurons in the CP and their results suggest a function of CP different from TS, with perhaps the auditory thalamus involved more in the integration of frequency-selective channels for processing more complex, wideband spectral features of natural or socially-relevant sound sources. Increases in egr-1 transcripts within the auditory thalamus of the Tungara frog have been shown to relate to a behavioral (locomotor) response to auditory stimuli which suggests the thalamus as a site for sensorimotor integration [75]. We found a greater difference in cFos response in the CP of social signal recipients vs. ambient noise compared to differences in lower auditory centers, suggesting possible higher-order auditory functions, e.g., [74]. The role of CP as a sensorimotor integration center in response to conspecific auditory signals in teleost fishes warrants further study [75].

cFos-ir Response in Vocal-Acoustic Circuitry/Nodes of SBN

Both the anterior (AT) and ventral (vT) tuberal hypothalamus comprise part of the midshipman descending vocal-motor system where vT is the most robust forebrain vocal stimulation site [76–78]. AT receives input from CP and receives strong projections from TS and is therefore also considered part of the ascending auditory system [9,19,67]. The differentially stronger input from the auditory system may explain, in part, the greater cFos response in AT versus vT to the social acoustic signal over ambient noise.

In order to assess the social relevance of a stimulus, integration of environmental cues and internal state must occur within the CNS [9,79,80] and the social behavior network (SBN) represents a suite of nuclei which may coordinate the production of an appropriate behavioral response to such cues [5–7]. The teleost AT and vT are thought to be partially homologous to the mammalian and avian ventromedial hypothalamus (VMH) and anterior hypothalamus (AH), respectively [6,8,81] which are designated nodes in the SBN. Recently, Maruska and colleagues [80] showed that levels of IEG transcripts (both cfos and egr-1) were elevated in AT and vT of African cichlid (Astatotilapia burtoni) males during social ascension from a subordinate to a dominant phenotype. In that study social ascension was triggered by the removal of an established dominant male from the environment, and the increase in IEG transcripts in AT and vT (as well as every other node of the SBN) was attributed to the perception of a social opportunity [80]. As midshipman are nocturnal, we tested type I
males at night when they typically court females [11] and we manipulated only their acoustic environment (playback of advertisement calls vs. ambient noise) during our experiments. Hearing another male’s advertisement call may be a signal that causes activation of AT within the neural circuit(s) responsive to a social opportunity in the context of courtship and reproduction. While type I males build and defend nests, their mate call is not typically displayed during direct agonistic encounters [14]. Alternatively, hearing another male’s mate call within a competitive social context could be perceived as a challenge and cause a neural response pattern associated with territoriality or competition. In mammals, cFos responses within the VMH have implicated this nucleus in neural circuits dedicated to both agonistic and mating behavior [82]. In a territorial Estrildid finch, the violet-eared waxbill (Uraeginthus granatinius), increased cFos-ir (as well as EGR-1-ir) neurons are found in the VMH and AH after exposure to a male conspecific [93]. In breeding condition, male European starlings (Sturnus vulgaris) behave territorially when in possession of a nest box from which to vocally court females, and have a higher baseline level of cFos-ir neurons in VMH and AH than do males without nest boxes [94]. Interestingly, the number of cFos-ir neurons in VMH and AH had a significant positive relationship with total number of songs produced by breeding condition males regardless of whether or not they possessed a nest box [94]. The ability to perceive social acoustic stimuli is thought to be adaptive only if it facilities an appropriate behavioral response [8]. Previously, it was demonstrated that “challenging” male Gulf toadfish with a playback of other male advertisement calls changes their internal physiological state (increases levels of circulating 11-ketosterosterone and cortisol), and causes an increase in both the rate and duration of a vocal response [23]. Our results suggest AT may play an important role for integrating the natural social acoustic environment with a subsequent vocal response [9,23]; however, we were unable to monitor vocalizations in response to mate-call playbacks in this experiment. Future studies will be needed to identify behavioral responses of males to social acoustic signals in this species.

cFos-ir Response in TH-ir Neurons in the TPa and LC

While circulating steroid hormones are documented to increase during playback of advertisement calls in male toadfish (see above), they are unlikely to be the only chemical responders to such a social challenge. Levels of the catecholamines DA and NE have been shown to increase in the brains of the lizard (Anolis carolinensis) during presentation of an actual [83] or simulated [86] opponent. Similarly, male Lincoln’s sparrows exposed to challenging songs of other males showed increased noradrenaline metabolites in the auditory forebrain and may be involved in the modulation of a motivated behavioral response to such a challenge [87]. DA has been implicated in motivated sexual behavior [36,88] including appetitive sexual behavior in the form of female directed vocalizations in zebra finches [35,89,90] and European starlings (Sturnus vulgaris) [91]. Additionally, much attention has been paid to DA’s involvement in how the female brain responds to conspecific competitors [23,100] or potential mates [13,15,94]. In midshipman fish, seasonal auditory plasticity in females is steroid-dependent and improves peripheral auditory encoding to better detect the male advertisement call [17]; however, the neurochemicals that potentially modulate the CNS in both females and males to mediate behavioral responses to social signals are less understood [101]. The catecholamine NE has been implicated in general arousal, selective attention, and neural encoding of salient sensory stimuli [26,102]. The locus coeruleus (LC) within the isthmus of the rostral hindbrain represents the primary ascending NEergic system in teleost fish [31,32,52,103], and its projection targets are highly conserved throughout vertebrates and include the auditory midbrain [27,31]. We report a greater percentage of TH-ir cells colocalized with cFos-ir within LC of males exposed to other male advertisement calls, which is consistent with results found in female zebra finches exposed to male courtship vocalizations [104]. We also report that cFos induction in the LC had a significant positive correlation with
number of cFos-ir neurons at all levels of the auditory and vocal-acoustic pathways that we analyzed. A positive correlation with percent colocalization in LC with cFos-ir neurons in DOri as well as TSnc, CP, AT, and vT, is consistent with NE’s involvement in neural response to social acoustic signals in a teleost. Future studies will be needed to study similarly demonstrate a neural response in LC to social acoustic signals in teleost fish. Overall our data support a conserved and integral role for catecholamines in vocal-acoustic social behavior across vertebrate taxa.

References


Acknowledgments

We thank Carol Vines, Gary Cherr, Kitty Brown, Peter Alderks and the Bodega Marine Laboratory for logistical support for this study, and Zachary Ghahramani for assistance with immunohistochemistry. We also thank two anonymous reviewers for helpful comments on the manuscript.

Author Contributions

Conceived and designed the experiments: PMF JAS. Performed the experiments: CLP MT DSK AAB RAM PMF JAS. Analyzed the data: CLP PMF JAS. Contributed reagents/materials/analysis tools: PMF JAS. Wrote the paper: CLP PMF JAS. 


