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RESEARCH ARTICLE

Directional sound sensitivity in utricular afferents in the toadfish *Opsanus tau*

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ABSTRACT

The inner ear of fishes contains three paired otolithic end organs, the saccule, lagena and utricle, which function as biological accelerometers. The saccule is the largest otolith in most fishes and much of our current understanding on auditory function in this diverse group of vertebrates is derived from anatomical and neurophysiological studies on this end organ. In contrast, less is known about how the utricle contributes to auditory functions. In this study, chronically implanted electrodes were used, along with neural telemetry or tethers to record primary afferent responses from the utricular nerve in free-ranging and naturally behaving oyster toadfish *Opsanus tau* Linnaeus. The hypothesis was that the utricle plays a role in detecting underwater sounds, including conspecific vocalizations, and exhibits directional sensitivity. Utricular afferents responded best to low frequency (80–200 Hz) pure tones and to playbacks of conspecific boatwhistles and grunts (80–180 Hz fundamental frequency), with the majority of the units (~75%) displaying a clear, directional response, which may allow the utricle to contribute to sound detection and localization during social interactions. Responses were well within the sound intensity levels of toadfish vocalization (approximately 140 SPL dB_{rms} re. 1 µPa with fibers sensitive to thresholds of approximately 120 SPL dB_{rms} re. 1 µPa). Neurons were also stimulated by self-generated body movements such as opercular movements and swimming. This study is the first to investigate underwater sound-evoked response properties of primary afferents from the utricle of an unrestrained/unanesthetized free-swimming teleost fish. These data provide experimental evidence that the utricle has an auditory function, and can contribute to directional hearing to facilitate sound localization.

KEY WORDS: Auditory, Hearing, Neural telemetry, Teleost, Utricle

INTRODUCTION

Sounds detected by the inner ear provide important cues for fishes to mediate fundamental behaviors such as predator and prey detection, and social interactions including territoriality and mating (Ladich and Myrberg, 2006; Myrberg and Lugli, 2006). The oyster toadfish, *Opsanus tau* Linnaeus, is a benthic ambush predator that inhabits inshore waters of the Eastern coast of the USA, and uses vocal communication in both aggressive and reproductive contexts (Fish, 1972; Gray and Winn, 1961; Maruska and Mensinger, 2009). For example, both male and female toadfish produce different types of grunt vocalizations that may function during agonistic interactions such as nest defense, competition for space, food or mates, or in distress/disturbance situations (Gray and Winn, 1961; Maruska and Mensinger, 2009). During the breeding season in early spring, males establish nesting sites and acoustically attract females by producing boatwhistle advertisement sounds via rapid contraction of sonic muscles surrounding the swim bladder. Spawning then ensues within the confines of the nest, and the male remains to guard the nest until the juvenile toadfish detach from the substrate several weeks later. Multiple spawning may result in several different clutches of eggs developing simultaneously within an individual male's nest (Gray and Winn, 1961; Gudger, 1910; Mensinger et al., 2003; Mensinger and Tubbs, 2006). While acoustic communication is crucial for reproduction in this species, there remains only limited information on how conspecific sounds are detected, localized and encoded by different components of the inner ear.

Many studies have examined the mechanisms involved in the production and reception of sounds in the vocal batrachoidid fishes (primarily *Opsanus* and *Porichthys* spp.), including investigations on sonic muscle properties and auditory physiology (see reviews by Amorim, 2006; Bass and McKibben, 2003). However, the mechanism by which female fish localize and choose males remains unknown. While terrestrial vertebrates use differential response times in sound reaching their auditory organs to localize sound (Popper and Fay, 2005), this is complicated in fishes by small inter-aural distances and the relatively rapid propagation of sound underwater. Recent studies in the plainfin midshipman show that these fish tend to follow the axes of local particle motion vectors produced by an underwater sound source to localize it (Zeddies et al., 2012). What remains unknown, however, is the relative involvement of the different otolithic end organs in the fish inner ear, for sound detection and localization.

The otolithic end organs in teleost fishes (saccule, utricle and lagena) serve gravistatic and auditory functions to encode linear particle motion. The saccule is the largest otolith and considered the primary auditory end organ in most fish (Popper and Fay, 1999). The response characteristics of saccular afferents have been studied across a wide variety of fishes including goldfish (Fay, 1978), midshipman (Sisneros and Bass, 2005), sleeper goby (Lu et al., 1998) and toadfish (Fay and Edds-Walton, 1997). The saccule is sensitive to linear acceleration and directionally sensitive to acoustic particle motion, functioning predominantly as a low frequency detector (60–1000 Hz). The range of saccular afferent frequency sensitivity in the toadfish encompasses the fundamental frequency of the male boatwhistle sound (~150–200 Hz) and grunt vocalizations (~50–250 Hz) (Edds-Walton et al., 1999, 2002).

The smaller utricle has received less attention and there is limited information on its potential role in directional hearing, having been examined in only a few of the more than 30,000 species of fishes. For example, utricular afferents showed directional responses to a single frequency (140 Hz) in the goldfish (Fay, 1984) and afferents in the sleeper goby *Dormitator latifrons* were directionally sensitive to pure tones from 50 to 400 Hz (Lu et al., 2004). However, neither

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the goldfish nor sleeper goby produces sounds for acoustic communication. Therefore, examining how the utricle might respond to underwater sounds in a vocal teleost such as the toadfish is important for understanding the evolution of auditory processing and vocal–acoustic signaling.

To test whether the utricle plays a role in sound detection and localization in the vocal toadfish, a neural telemetry tag was used that allowed for sound presentation to free-ranging and naturally behaving fish while simultaneously recording single neuron responses from the utricular nerve. The goals of this study were to determine whether the toadfish utricle (1) is sensitive to sound, (2) has directional sensitivity and (3) can encode conspecific vocalizations. The majority of recent studies on sound sensitivity in toadfish have been performed on a shaker table (Edds-Walton and Fay, 2008) that, while allowing precise correlation of neural activity with fine linear movements, necessitates using anesthetized and/or restrained fish. Even low concentrations of anesthetic can depress nerve sensitivity (Palmer and Mensinger, 2004) and as the otolithic organs also encode linear acceleration, it is unclear what impact self-generated movement might have on auditory function.

Our results using neural telemetry in unrestrained/unanesthetized individuals demonstrated that the utricle of the oyster toadfish does respond to underwater sound playbacks, including conspecific vocalizations, and is directionally sensitive. These data provide important insights on the function of the utricle end organ in naturally behaving fish. Further, our results provide support for an auditory function of the utricle in a vocalizing teleost that relies on acoustic communication during agonistic and reproductive contexts.

RESULTS

The tether and telemetry tag both provided effective means to monitor neural activity, with multiple units often distinguishable based on amplitude and waveform shape in each implant (Fig. 1). Toadfish normally spend long periods of time motionless inside sheltered habitats with occasional brief forays limited mainly to foraging. Pre- and post-operative fish displayed similar behavior, and neither the tag nor the tether restricted movement, inhibited respiration or precipitated behavior to dislodge the devices.

While the spontaneous discharge rates of the initial implants often were slightly elevated while the fish were on the table [mean±s.e.m. 19.6±2.3 spikes s^{-1}, range 0 (silent) to 54 spikes s^{-1}, N=45 afferents in 20 fish], by the time the fish were placed in the experimental aquarium, these rates attained a steady state that remained consistent over consecutive days (12.5±2.0 spikes s^{-1}; initial versus steady state, paired t-test, t=2.95, d.f.=17, P=0.009). The majority of utricular afferents (N=45 afferents in 20 fish) showed irregular-type discharge activities [mean interspike interval (ISI) 0.12±0.02 s, coefficient of variation (CV) 0.74±0.03], with a small percentage (~12%) that were silent with resting rates <1 spike s^{-1}. There were no afferents, however, that met the strict criteria for a regular discharge pattern (CV<0.40), although ~17% of neurons had CV values between 0.40 and 0.60.

In addition to anatomical landmarks, the recordings were confirmed to originate from utricular afferents by subjecting all fish to horizontal and vertical movements of the vibration isolation table prior to sealing the craniotomy. All neurons, with the exception of the rare putative efferent fiber, responded in phase with horizontal table movements, but were relatively insensitive to vertical motion (Fig. 2). This response is predictive of neurons innervating the horizontally positioned utricular macula. Tank-mounted accelerometers showed that afferents in all the fish analyzed (N=20) were sensitive during horizontal table oscillation displacements of 1–3 cm at approximately 0.5–3.0 Hz. As expected, afferents were only excited during a portion of the stimulus cycle and remained silent or showed reduced activity as the table returned to its original position (Fig. 2C).

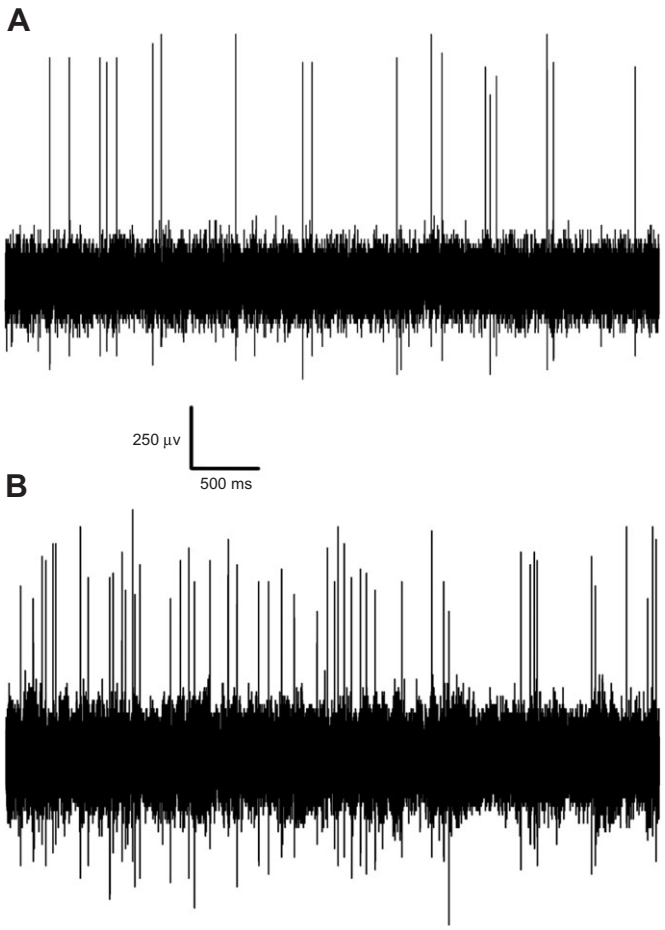

Fig. 1. Spontaneous neural activity recorded from a single primary afferent in the utricular nerve following microwire electrode implantation. (A) Telemetry signal from a tethered fish and (B) neural activity in the same fish 24 h earlier (approximately 30 min after electrode implantation) to illustrate the high fidelity signal using both methods. Initial spontaneous activity was usually higher immediately after implantation, but typically attained a steady baseline within 2 h of placement in the experimental aquarium.

Utricular afferents were also stimulated during the fish's natural ventilation cycle (Fig. 3). Although opercular movement was clearly visible during ventilation, forward or side to side displacement of the toadfish body was not readily discernible during breathing. In the large adult fish used in this study, breathing movements rarely displaced the quiescent toadfish more than ±2 mm and, in many cases, utricular neurons fired without visible movement, demonstrating the high sensitivity of these fibers to small displacements. The breathing cycles of six fish were examined for utricular activity during and between opercular movements. The average time between the start of opercular movements was 8.62±1.05 s (approximately 7 breaths min^{-1}) with the opercular motion lasting an average of 1.47±0.11 s. Utricular afferent activity was significantly elevated during gill movement with firing rate increasing to 10.96±1.73 spikes s^{-1} versus 1.3±0.11 spikes s^{-1} during the stationary phase (paired t-test, P<0.001).

Sustained forward movement of several seconds during either natural or evoked swimming (N=4 fish) led to continuous and

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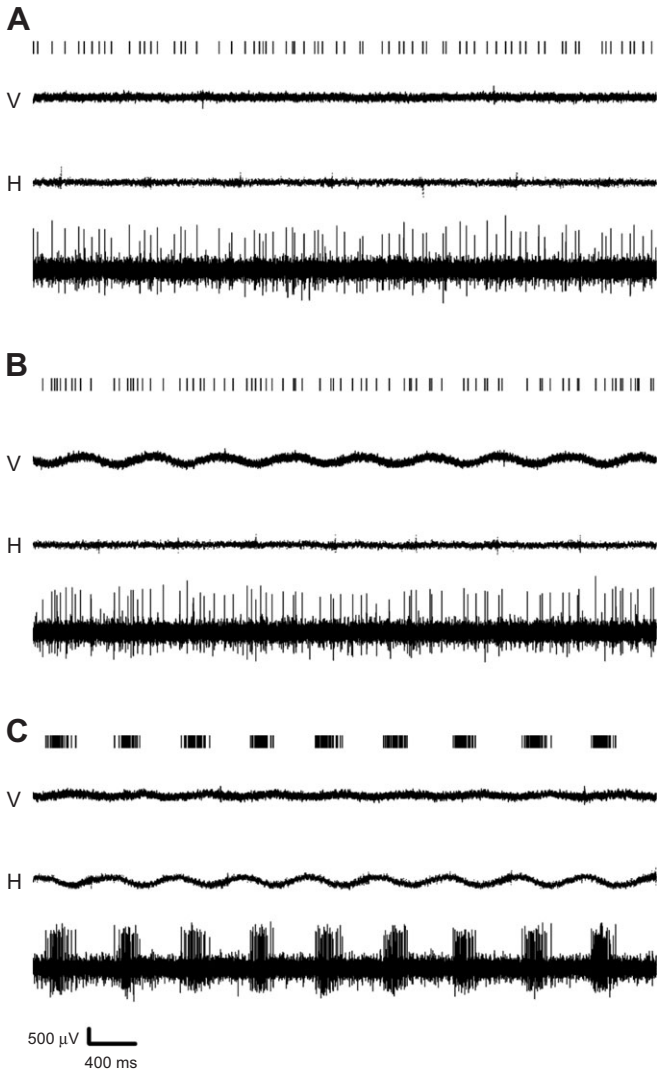

Fig. 2. Utricular afferents responding to low frequency linear motion. An *x*- and *y*-axis accelerometer was placed on the toadfish stereotactic tank and the vibration isolation table was oscillated at approximately 1.5 Hz. A–C show, from top to bottom: vertical marks representing discriminated individual action potentials, vertical axis accelerometer voltage (V), horizontal axis accelerometer voltage (H) and neural activity from the utricular nerve. Each panel is 5 s in duration. (A) No movement; (B) vertical movement; (C) horizontal movement.

elevated discharge rates in utricular afferents during the relatively brief movements (<2 s). For example, a toadfish that had a resting discharge of 1.36±0.23 spikes s^{-1} showed a significant increase in spike rate during both ventilation (13.10±2.09 spikes s^{-1}) and swimming (64.0±8.02 spikes s^{-1}) (ANOVA, P<0.001).

Utricular neurons were also responsive to playbacks of underwater sound. Fig. 4 shows the firing characteristics of a single afferent neuron in response to a tone stimulus of 120 Hz located 90 deg to the left of the toadfish. The hydrophone was positioned directly over the head, approximately 80 cm from the speaker to monitor the sound as it reached the toadfish inner ear. The neuron consistently fired during sound presentation, and increasing stimulus intensity resulted in greater firing rates. For example, there was an average 200% increase in firing rate between resting rate (20.22±4.23 spikes s^{-1}) and the response to tone playbacks at 10 dB above threshold at best frequency (62.10±8.63 spikes s^{-1}) for

Fig. 3. A utricular primary afferent stimulated by respiratory activity. The waveform (bottom) represents activity from a single afferent recorded via neural telemetry from a stationary toadfish with a respiration rate of approximately 0.25 Hz. Vertical marks above the waveform represent discriminated individual action potentials and the inverted triangles indicate the initiation of opercular contraction.

individual afferent neurons (paired *t*-test, P<0.001, N=9). Utricular afferents were most sensitive to relatively low frequency sounds between 80 and 160 Hz (mean best frequency 113.8±5.6 Hz, N=9 fish; frequencies lower than 80 Hz were not testable with the underwater speaker).

Fig. 5A shows the tuning curves from three representative afferent fibers that demonstrated broad sensitivity within this range before becoming less sensitive as frequencies increased over 200 Hz. Mean sound pressure level (SPL) thresholds ranged from ∼125 to 150 dB_{rms} re. 1 µPa (where rms is root mean square) over the frequency range tested (80–400 Hz) (Fig. 5B).

Utricular afferents were also responsive to playbacks of toadfish vocalizations, including agonistic grunts and reproductive boatwhistles (Fig. 6). Utricular afferents were responsive to boatwhistle presentations at approximately the same SPL observed for the pure tones. A fiber that showed a background discharge of 24 spikes s^{-1} increased its firing rate above background in response to a series of 10 boatwhistle playbacks (292.0±3.0 ms duration, range 253–335 ms) at 127 dB_{rms} re. 1 µPa, reaching rates of 80 spikes s^{-1} above 132 dB_{rms} re. 1 µPa (Fig. 7).

Most utricular neurons (75%) displayed directional sensitivity to sound (0–180, 45–225, 90–270 or 135–315 deg) using either vector strength or spike rate as the measured criterion (N=12; Fig. 8). Of these, the majority (45%) showed best sensitivity along the 0–180 deg axis (Fig. 8B), followed by 27% along the 90–270 deg axis, 18% along the 135–315 deg axis and 9% along 45–225 deg axis (Fig. 8A). The non-directional (or omnidirectional) neurons still responded robustly to sound playbacks; however, there was no clear directionality (Fig. 8C). Fig. 9 shows the increase in firing rate (spikes s^{-1}) for a directional (45–225 deg axis) utricular afferent responding to playbacks of a 100 Hz tone at three different SPL intensities.

DISCUSSION

A combination of recording techniques including a long tether (free-ranging) and neural telemetry tag (free-swimming) were used to characterize the responses of single utricular primary afferents to self-generated movements such as respiration and swimming, as well as responses to underwater sound presentation (tones and conspecific vocalizations). Recording fidelity was similar between the telemetry tag and tether, with most neurons responsive to low frequency movement in the horizontal plane,

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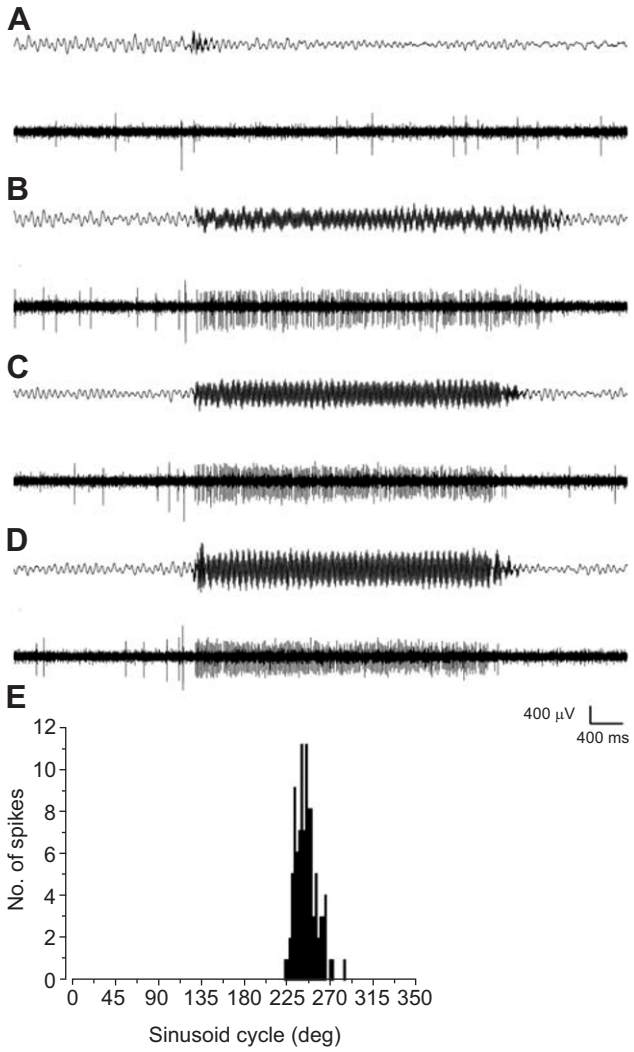

Fig. 4. Activity of a utricular primary afferent monitored during 120 Hz tone stimulation. The underwater speaker was located 90 deg from the left side of the toadfish head at a distance of 80 cm. In A–D, the top trace is the voltage amplitude from the hydrophone located directly above the toadfish's habitat and the bottom trace is the telemetry-recorded waveform of action potentials from the utricular nerve. (A) The afferent at rest without stimulation; (B–D) successive increases in stimulus strength (increments of ~5 dB). (E) Histogram of action potentials versus location (deg) in the pure tone stimulus when the unit fired to illustrate strong phase locking.

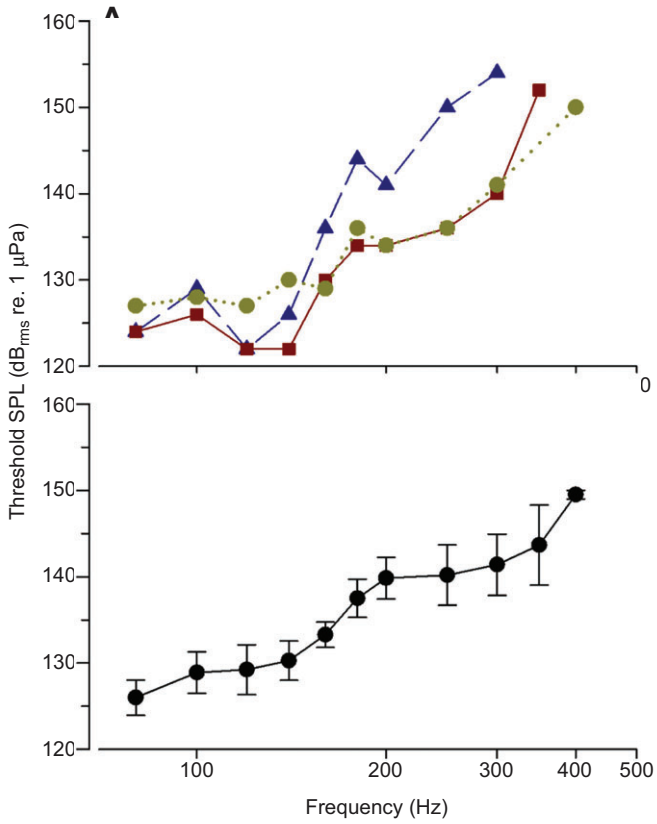

Fig. 5. Tuning curves for utricular primary afferents in the toadfish. The sound intensity (SPL, sound pressure level) needed to evoke the threshold criterion response is plotted versus sound frequency for utricular afferents in the toadfish. (A) Threshold tuning curves for three single neurons in different individual toadfish. (B) Mean±s.e.m. threshold for toadfish (*N*=12) in which tuning curves were generated.

and showed directional sensitivity to sound stimuli including playbacks of toadfish vocalizations such as grunts and boatwhistles. This is the first study to record underwater sound-evoked primary afferent responses from the utricle in a free-swimming fish, and the results indicate that the utricle can encode directional information, and therefore may play a role in sound localization in the toadfish.

Most previous investigations on auditory sensitivity have used anesthetized and/or restrained fish that are unable to move naturally, and therefore it remains unclear how fish integrate simultaneous input from self-generated motion and external sound stimuli. Additionally, even low doses of the anesthetic MS-222 can depress neural sensitivity in fish (Palmer et al., 2005; Palmer and Mensinger, 2004). The chronically implanted electrodes allowed neural activity to be monitored during normal toadfish movement, providing new insights into how otolithic end organ neurons respond in naturally behaving fish. The telemetry tag and tether did not noticeably impact toadfish behavior and previous studies demonstrated that the magnetic field does not affect neural activity or behavior in the toadfish (Mensinger and Deffenbaugh, 1998, 2000; Palmer et al., 2005). The sensitivity of the anterior lateral line to mechanical stimuli was restored within 90 min of anesthetic withdrawal (Palmer and Mensinger, 2004), and, therefore, allowing a minimum of 90 min following the discontinuation of anesthesia should have eliminated the effects of MS-222. Normal ventilation rates and equilibrium returned within 30 min of anesthetic withdrawal, swimming resumed within 2 h of being placed in the experimental tank, and fish often would resume feeding within 24 h of surgery.

Utricular neurons were quite sensitive to horizontal but not vertical movements of the toadfish. Small, horizontal oscillations (<1 cm) of the vibration isolation table quickly evoked increased firing in utricular afferents while vertical movements typically did not affect discharge rates. Swimming also stimulated increases in action potentials, often detected as bursts of neural activity associated with body movements. However, as the toadfish is an ambush predator, its normal behavior is to remain motionless in its habitat for long periods. Although ventilation has been shown to stimulate neurons innervating the lateral line system (Montgomery and Bodznick, 1994), there have been no previous reports that breathing in stationary fish also influences auditory or vestibular systems. Although the opercular movements were readily visible, they did not appear to produce horizontal body displacements of more than 1 or 2 mm. Thus, any role of the utricle in sound detection must take into account

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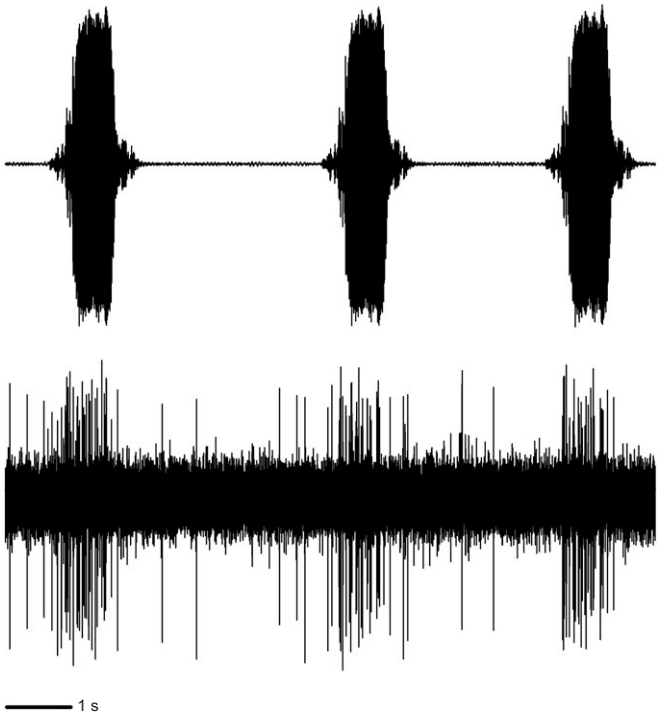

Fig. 6. Response of a single utricular afferent to playback of toadfish boatwhistle vocalizations. The top trace is the playback of three toadfish boatwhistles presented via the underwater speaker and recorded by the hydrophone at the toadfish head, while the bottom trace is the waveform of the utricular neural activity recorded from a tethered toadfish. The boatwhistle intensity (SPL) was approximately 135 dB_{rms} re. 1 µPa.

self-stimulation from respiratory activity and/or swimming. Filtering self-generated sensory signals often is necessary to remain aware of external cues and can be accomplished in higher order processing centers (Montgomery and Bodznick, 1994; Requarth and Sawtell, 2011). Anti-Hebbian synaptic plasticity in cerebellum-like circuits in elasmobranchs and weakly electric fish can cancel the electrical input generated by the fish movements (Bell et al., 1997; Bodznick et al., 1999; Montgomery et al., 1995).

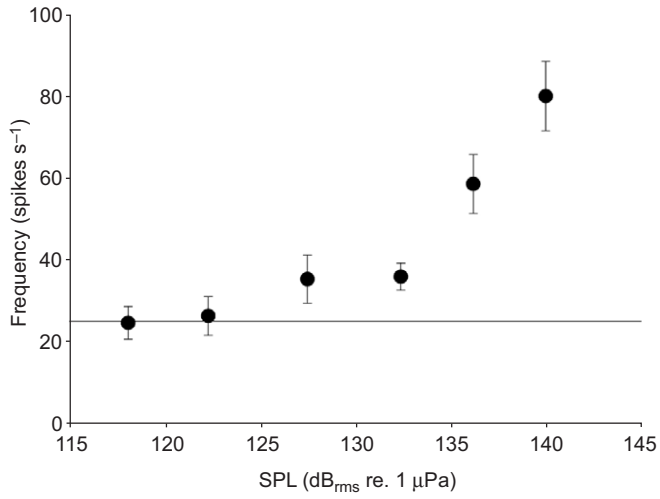

Fig. 7. Utricle response versus SPL for playbacks of toadfish boatwhistles. The response of a utricular afferent with spontaneous discharge of 25.0±1.3 spikes s^{-1} (solid line) is plotted versus presentations of 9–10 boatwhistles at different intensity levels. Mean±s.e.m. frequency is plotted.

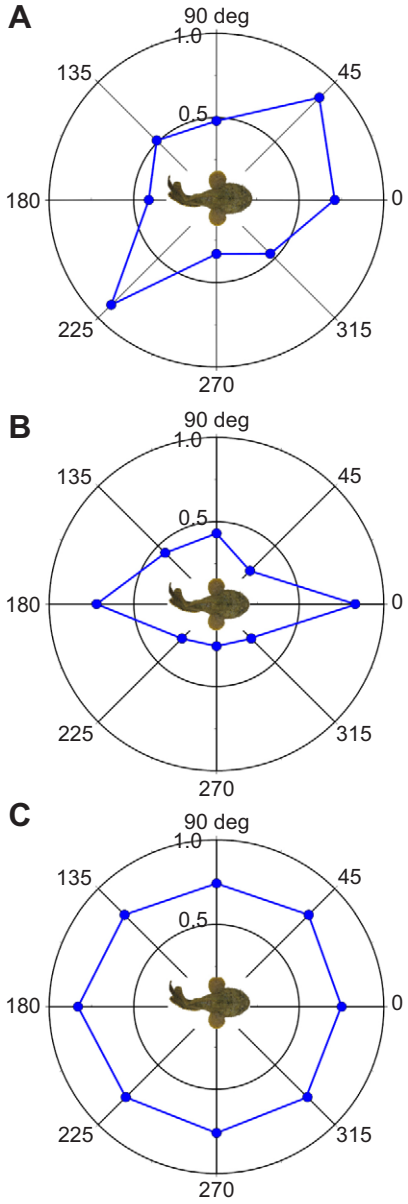

Fig. 8. Diversity of directional responses of utricular primary afferents in the toadfish. Polar plots of neural responses using vector strength analysis from three representative utricular primary afferents. (A) Directional afferent with best sensitivity along the 45–225 deg axis, (B) directional afferent with best sensitivity along the 0–180 deg axis and (C) afferent with omnidirectional sensitivity. Plots were constructed from recordings at the best frequency of each afferent at 5 dB above threshold. The distance from the central origin to each data point represents the vector strength, or coefficient of synchronization, at each angle. Dorsal view of a toadfish is shown in the center of each plot and 0–180 deg represents the rostro-caudal fish axis.

Lu et al. (2004) found that most utricular neurons in the sleeper goby were responsive to linear accelerations less than 100 Hz with characteristic frequencies distributed from 50 to 400 Hz with a mode of 80 Hz. The underwater speaker in our study precluded testing frequencies less than 80 Hz; however, the toadfish utricular neurons were most sensitive from 80 to 200 Hz with decreasing sensitivity at higher frequencies. This sensitivity corresponds with the fundamental frequency of toadfish grunts (80–120 Hz) and male boatwhistles (100–200 Hz). The intensity of toadfish vocalizations was reported to be 140 dB (Tavolga, 1971). The afferents in our

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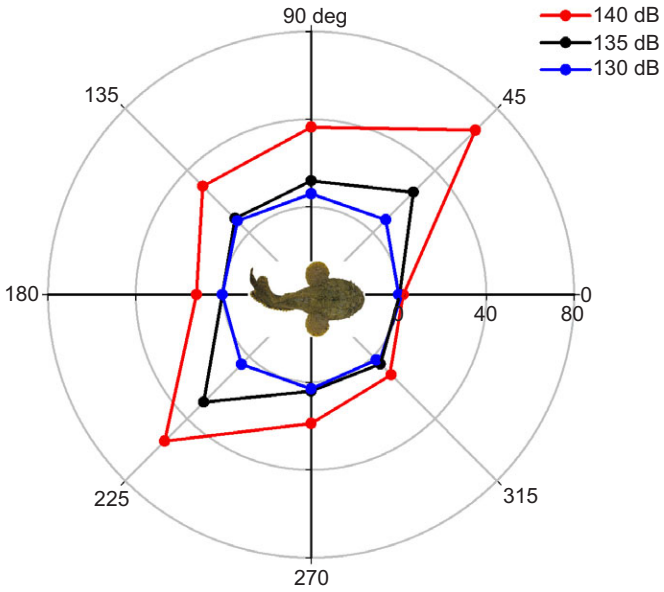

Fig. 9. Afferent response to increased sound intensity. Polar plot of directional (45–225 deg axis) neural responses (spikes s^{-1}) from a single utricular afferent to playbacks of a 100 Hz tone at three different SPL intensities (140, 135 and 130 dB_{rms} re. 1 µPa). Dorsal view of a toadfish is shown in the center of the plot and 0–180 deg represents the rostro-caudal fish axis.

study showed sensitivity to pure tones at approximately 120 dB and to vocalizations at approximately 125 dB. Thus, not only is the utricle sensitive to low frequency sound but also it is well designed for detecting the frequencies and intensities of toadfish vocalizations used for intraspecific communication. One requirement for sound localization, however, is that the end organ should exhibit directional sensitivity to an underwater sound source. Our data show that the majority of utricular neurons did exhibit directional sensitivity, suggesting the utricle may be involved in sound localization, particularly in the azimuth. Many neurons showed strong directionality along the 0–180 deg axis and 90–270 deg axis, while a few fibers appeared omnidirectional. Although only a small percentage of afferents were characterized as predominantly sensitive to 45–225 or 135–315 deg, all neurons did respond along these axes, and it is possible that afferents responding optimally to these directions may not have been accessible in the portion of the nerve available for implant. These differences in directional responses are likely due to afferents innervating different populations of hair cells within the utricular macula.

Several additional recorded neurons were acceleration sensitive (e.g. table or fish movement), but relatively insensitive to sound presentations via the underwater speaker, suggesting dichotomy in utricular hair cells with some hair cells functioning primarily as low frequency vestibular and not auditory sensors. Alternatively, as these cells were not tested for sound sensitivity between 5 and 80 Hz, these may be representative of the lower frequency fibers found in the utricle of the sleeper goby (Lu et al., 2004).

The ability of fish to localize sound sources is complicated by small inter-aural distances and the high speed of sound underwater. The saccule has been implicated as the main end organ of hearing and is certainly the largest otolith in toadfish. However, the caudal ends of the bilaterally positioned saccules are in close proximity, and, even in adult fish, sound arrives at the posterior of each end organ virtually simultaneously. The smaller utricles, in contrast, are rostral to the saccules and in large, adult toadfish, are separated by distances of 1–3 cm. Whether this spacing provides a sufficient delay to localize sounds based on inter-aural time differences remains to be determined.

What is clear, however, is that body movements and normal ventilation can also stimulate the utricle, and while these cyclic movements may be filtered in higher order processing centers (Montgomery and Bodznick, 1994), the ability to hear and/or find the sound source may be compromised by self-generated movement. While male toadfish remain relatively stationary during advertisement calling, female fish swim to find suitable males. Swimming movements can cause maximal excitation of utricle afferents and the ability to pinpoint sound sources during these forays may be compromised. Although observations of female fish approaching males from a distance are complicated by the poor environmental visibility, one would predict that, if the utricle is important in localizing sound, the female may need to alternate swimming with stationary pauses to assist in locating the sound. Spontaneous toadfish movements in outdoor ponds and large tanks suggest that typical toadfish ‘swimming’ does consist of short ‘legs’ of less than 1 m with intermittent pauses, rather than sustained bursts of long-distance travel. While this behavior is more likely to have evolved to minimize alerting potential prey or avoiding predation outside of their protective habitats, it may also allow the fish to sample its acoustic environment without the added complications of self-generated movement.

This study is the first to investigate underwater sound-evoked response properties of primary afferents from the utricle of a teleost fish that relies heavily on acoustic communication for intraspecific behaviors. Further, it is also the first study to examine thresholds and directional responses of primary afferents in the utricle of unrestrained/unanesthetized free-swimming fish, using implantable electrodes, tethered recordings and neural telemetry. Our data provide experimental evidence that the utricle has an auditory function in the toadfish, and that it can contribute to directional hearing possibly to facilitate sound localization. The high responsivity of the utricle in the horizontal plane suggests it may function in detecting particle motion in azimuth, while the more vertically oriented saccule and lagena better detect particle motion in elevation. For the benthic-dwelling toadfish, sound detection in the horizontal plane is likely extremely important for detecting sounds generated by conspecifics, predators and prey. Further studies are needed, however, to determine the relative role of each of the different otolithic end organs and how they contribute to sound localization in fishes.

MATERIALS AND METHODS

Adult toadfish of both sexes (N=16 male, N=4 female; mean±s.d. body mass 494.4±114.6 g, standard length 24.8±2.3 cm) were obtained from the Marine Biological Laboratory (MBL), Woods Hole, MA, USA. Fish were housed in large flow-through seawater tanks maintained at 20°C and fed squid and bait fish. All animal care and experimental procedures conformed to institutional animal care protocols.

Microwire electrodes

To record single neuron responses in free-swimming toadfish, microwire electrodes were implanted into the utricular nerve. The electrodes consisted of three strands of insulated 20 µm-diameter 10% platinum/iridium wire (Sigmund Cohn Corp., Mt Vernon, NY, USA), and were custom fabricated for each implantation. Each microwire strand was affixed to multistranded wire (25 µm diameter) with silver paint (Silver Print Paint, GC Electronics, Rockford, IL, USA). The multistranded wire was attached to silver wire (320 µm) that terminated into a multipin underwater connector. The anterior portion of the microwire was threaded through a 1 mm length of polyimide

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tubing (180 µm outer diameter; A-M Systems Inc., Carlsborg, WA, USA) to maintain the multiple recording sites in proximity. Exposed wire/connections were encased in medical device adhesive (Loctite 3341; Henkel Loctite Corp., Rocky Hill, CT, USA) and cured with ultraviolet light (ELC no. 660; Electro-lite Corp., Danbury, CT, USA). The impedance of each electrode was determined with an impedance-test unit (FHC; Bowdoinham, ME, USA) using 1 kHz input frequency and ranged between 0.5 and 1.2 MΩ.

Electrode implants

Fish were anesthetized by immersion in 0.005% tricaine [3-aminobenzoic acid ethyl ester (MS-222); Sigma, St Louis, MO, USA], immobilized with an intramuscular injection of 0.01% pancuronium bromide solution (600 µg kg^{-1}; Sigma) and placed into a small, acrylic stereotactic tank on a vibration isolation table. An incision was made through the dorsal musculature overlying the sagittal crest, and the muscle retracted. A small craniotomy was performed lateral to the sagittal crest and posterior to the transverse crest to expose the utricular nerve. The microwire electrode was inserted into the nerve approximately midway between the utricular otolith and brain. Extracellular potentials were differentially amplified (Dagan, Minneapolis, MN, USA) and monitored on a portable computer using Chart5 for Windows software (AD Instruments, Colorado Springs, CO, USA). The two recording channels that provided the highest fidelity signal and sufficient signal-to-noise ratio (~2–5:1) in response to horizontal movements of the vibration isolation table were chosen for the experiments. In some cases, a three-channel accelerometer was affixed to the surgical tank to correlate neuron responses with table movements. Once a candidate neuron(s) was located, the fish was left undisturbed for 30 min to ensure recording stability. Cyanoacrylate gel (Pacer Technology, Rancho Cucamonga, CA, USA) was then used to affix the electrode to the skull and seal the craniotomy. The muscle was restored to its original position, and the muscle, fascia and epidermis were individually sutured to provide a watertight seal over the craniotomy and around the transdermal electrode lead. At this point, the electrode lead was connected to either a cylindrical (38×15 mm diameter) neural telemetry tag (Palmer et al., 2005) or a long, thin flexible tether (~2.0 m).

The telemetry tag was part of an inductive telemetry system consisting of the transmitter tag and receiver coils. The neural signals were transmitted as a frequency-modulated magnetic field (90 kHz carrier, 20 kHz bandwidth), which was detected by receiver coils embedded in a recharging habitat and stage (RECHABS). The RECHABS consisted of a cylindrical PVC habitat (12 cm internal diameter×30 cm; wall thickness 6.6 mm) that opened onto an octagonal stage (16 cm per side), and served to receive the telemetry signal and recharge the tag. Hydrophone recording with or without the habitat showed no difference in sound intensity. Telemetry and recharging was possible whenever the fish was within the footprint of the RECHABS up to an elevation of approximately 15 cm above the stage. The tag was fully powered in less than 30 s and provided telemetry for up to 20 min between charging. In other cases, the transdermal lead was connected via a waterproof connector to a long, thin three wire cable (~2.0 m) that terminated into the head stage of the amplifier outside the tank. Sufficient slack remained in the cable to allow the toadfish to freely move around the aquarium.

Immediately after surgery, the toadfish was placed in an opaque round fiberglass tank (~1 m diameter) with a water depth of 30 cm (salinity, 30–31 ppt; temperature, 20–22°C) and left undisturbed for a minimum of 90 min, a time previously shown to eliminate any effects of anesthesia on neural recordings (Palmer and Mensinger, 2004). A University Sound UW-30 speaker (frequency response 80 Hz to 10 kHz) was suspended vertically in the water column approximately 80 cm from the fish, and a hydrophone was placed directly above the toadfish's head at the approximate position of the utricular end organ.

Sound presentation

Pure tones and previously recorded male toadfish vocalizations were used as auditory stimuli and transmitted through a Speco PAT-20TB marine amplifier to the UW-30 speaker. The front of the RECHABS cylinder habitat was maintained 80 cm from the speaker and fish were presented with sounds only while in the habitat with their head facing out. As the fish were free to move, small displacements inside the RECHABS of ±5 cm from the opening and/or ±5 deg left or right were possible and allowed. However, if fish exited the habitat or retreated further than 5 cm into the habitat, the experiment was suspended and the fish gently repositioned in the cylinder. The habitats were rotated in 45 deg increments relative to the speaker (0, 45, 90, 135, 180, 225, 270, 315 deg) to test for directional sensitivity with the distance from the front of the habitat to the speaker remaining constant (i.e. the otoliths remained the same distance from the speaker). Thresholds were determined for each test frequency along the axis of best directional sensitivity by starting with a supra-threshold intensity followed by decreasing intensities in 3–5 dB steps until the afferent no longer responded to the stimulus (see threshold criteria in 'Neural recordings', below). A calibrated hydrophone (Bruel & Kjaer 8103 or High Tech HTI-94) was positioned above the fish's head during all experiments to record the sound stimulus reaching the toadfish. Relative SPL was calculated for each frequency and intensity by measuring the rms voltage at the position of the fish's head and converted to SPL in dB_{rms} re. 1 µPa.

Respiratory activity was monitored by placing a wire electrode on the fish's operculum or a hydrophone in line with the excurrent. Both methods reliably tracked the respiration cycle and changes in neural activity were correlated with each cycle. Swimming movements were monitored by an observer and noted on the data acquisition system. The initiation of swimming was characterized by a small movement artefact in the electrode trace and a significant increase in neural activity during movement.

Neural recordings

Single and multiunit recordings were amplified (×1000; Dagan Ex-1), filtered (300 Hz to 3 kHz) and recorded onto a computer using a Powerlab AD interface and Chart5 software. Spontaneous (resting) firing rates were recorded for each neuron and used to generate ISI histograms with 2 ms bins. A total of 200–500 spikes of spontaneous firing data were recorded for each neuron prior to sound stimulation. The CV, a dimensionless ratio of standard deviation to mean spike interval, was also calculated for each afferent to estimate relative variability in resting discharge patterns. Neurons with spontaneous activity were classified as regular (normal distribution; CV≤0.40) or irregular (Poisson-like distribution; CV>0.40) based on the shape of their ISI histogram and CV values. Following recordings of spontaneous activity, each afferent was tested for directional sensitivity and threshold at each test frequency as described above.

Neural responses to tones were quantified for vector strength (VS or synchronization coefficient, R) and evoked spike rates across the entire stimulus cycle. Spike rates for directional responses were expressed as the maximum evoked spike rate minus the mean resting rate for each neuron (e.g. peak-DC). VS was calculated according to Goldberg and Brown (1969) and is a measure of the degree of phase locking to a periodic signal determined by the mean vector length for circular distribution of spikes over the stimulus period. VS varies from zero (random distribution; no phase locking) to one (all spikes in the same bin; strong phase locking). The degree of phase locking (VS) was determined to be a better predictor for auditory frequency encoding among vertebrates than maximum evoked spike rates for frequencies ≤1 kHz (Fay, 1978, 1982, 1994; Javel and Mott, 1988; Sisneros and Bass, 2003).

The significance of phase locking was determined by the calculation of the Rayleigh statistic, Z, which is defined as $R^2 \times N$, where R is the coefficient of synchronization (or vector strength) and N is the total number of spikes sampled. The probability of observing $Z \geq 4.5$ by chance is 0.01 (Batschelet, 1981); thus, responses with $Z \geq 4.5$ were considered significantly phase locked. Threshold was defined at the lowest intensity to evoke an increase in spike rate above spontaneous activity, or a significant Z-value (≥4.5) as described in other studies (Lu and Fay, 1993; Maruska and Tricas, 2009). Threshold was determined for the following frequencies (80–400 Hz; 20 Hz increments from 80 to 200 Hz and 50 Hz increments from 200 to 400 Hz). Directional responses for each individual neuron were calculated at the same supra-threshold stimulus strength (~5–10 dB above threshold) at each of the eight different stimulus orientations and examined as both spike rate (spikes s^{-1}) and vector strength.

Data analysis

Neural activity was recorded for up to 4 days post-implantation (range, 2 h to 4 days) and stored on a portable computer using Chart5 software and analyzed offline with Spike2 software (Cambridge Electronic Design, Cambridge, UK). Although the microwires often yielded multiunit activity, neuron discrimination was usually limited to one or two units that yielded the greatest amplitude and had clearly distinguishable waveforms above the noise level. To verify that the same afferent(s) was consistently recorded during an experiment, individual fibers were distinguished using waveform analysis (Spike2) in addition to spike amplitude. All statistical analysis was performed using GraphPad Software (San Diego, CA, USA) or SigmaStat for Windows version 3.10 (Systat Software, Inc., Richmond, CA, USA). All data represent means±1 s.e.m. unless otherwise indicated.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.P.M. performed the majority of the experiments and data analysis. The two authors contributed equally to the study design and writing of the manuscript.

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