Neuronal Encoding of Ultrasonic Sound by a Fish

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1Department of Biology and 2Neuroscience and Cognitive Science Program, University of Maryland, College Park, Maryland 20742; 3National Museum of Natural History, Smithsonian Institution, Washington, DC 20560; and 4RWTH Aachen, 52074 Aachen, Germany

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Plachta, Dennis T. T., Jiakun Song, Michele B. Halvorsen, and Arthur N. Popper. Neuronal encoding of ultrasonic sound by a fish. J Neurophysiol 91: 2590–2597, 2004. First published January 28, 2004; 10.1152/jn.01200.2003. Many species of odontocete cetaceans (toothed whales) use high-frequency clicks (60–170 kHz) to identify objects in their environment, including potential prey. Behavioral studies have shown that American shad, Alosa sapidissima, can detect ultrasonic signals similar to those of odontocetes that are potentially their predators. American shad also show strong escape behavior in response to ultrasonic pulses between 70 and 110 kHz and can determine the location of the sound source at least in the horizontal plane. The present study examines physiological aspects of ultrasound detection by American shad and provides the first insights into the neural encoding of ultrasound signals in any nonmammalian vertebrate. The recordings were obtained by penetration through the cerebellar surface. All but two units responded exclusively to ultrasound. Ultrasound-sensitive units did not phase-couple to any stimulus frequency. Some units resembled the response of constant latency neurons found in the ventral nucleus of the lateral lemniscus of bats. We suggest that ultrasonic and sonic signals are processed along different pathways in the cerebellum and hindbrain. All but two units responded exclusively to ultrasound. Ultrasound-sensitive units did not phase-couple to any stimulus frequency. Some units resembled the response of constant latency neurons found in the ventral nucleus of the lateral lemniscus of bats. We suggest that ultrasonic and sonic signals are processed along different pathways in the cerebellum and hindbrain.

INTRODUCTION

Odontocete cetaceans (toothed whales) use audition to obtain information about their environment and potential prey and for social communication (Au 1993; Tyack and Clark 2000). They use short clicks (50–500 μs) to echolocate and longer whistles for communication (Au 1993; Norris et al. 1994). Clicks have peak frequencies between 120 and 140 kHz in the harbor porpoise (Phocoena phocoena) and between 70 and 130 kHz in the bottlenose dolphin (Tursiops truncatus) (Mohl and Andersen 1973; Sigurdson 1997), with signal levels ≤228 dB re: 1 μPa at 1 m (Au and Moore 1984).

Based on earlier studies of fish hearing (summarized in Fay 1988), it was assumed that fish do not detect ultrasound. It has recently been shown, however, that these frequencies are within the hearing range of several species of clupeid fishes such as herrings, sardines, anchovies, and American shad (Alosa sapidissima) (Mann et al. 1997, 1998, 2001).

In a behavioral study, American shad showed rapid directional escape behavior to signals from 70 to 110 kHz (Plachta and Popper 2003). The response pattern depended on stimulus amplitude and frequency and paralleled those of noctuid moths and other insects to their echolocating bat predators (Hoy 1992; Schulze and Schil 2001).

All clupeids, including American shad, have a highly specialized prootic bulla closely associated with the utricle of the inner ear (Allen et al. 1976; Best and Gray 1980; O’Connell 1955). This bulla is divided into two parts by a thin membrane that has air on one side and fluid on the other. The air-filled chamber is connected via a thin tube to the abdominally located swim bladder of the fish.

Unlike all other vertebrates (including all fish studied to date) that have one continuous utricular sensory epithelium (Platt 1984), the clupeid utricular epithelium is divided into three different sections: the anterior, posterior, and middle maculae (O’Connell 1955). The membrane extending from the prootic bulla is connected to the middle macula by a thin membrane (O’Connell 1955). Mann et al. (1997, 1998, 2001) hypothesized that this bulla-utricle connection is involved with ultrasound detection and a recent behavioral and morphological study strongly supports this suggestion (Higgs et al. 2004).

Although it is likely that American shad detect ultrasound using the ear, this remains to be demonstrated electrophysiologically. Thus the purpose of the present study was to investigate the neuronal response of American shad to pure-tone ultrasound stimuli by recording neuronal responses in the cerebellum and hindbrain to get an overview of the nature of responses to such signals. We recorded and identified responding units at different levels of the ascending and descending pathway. Most importantly, we considered whether or not sonic and ultrasonic (defined herein as frequencies below and above 20 kHz, respectively) information is processed through the same neurons and whether these units show characteristics similar to those found in other ultrasound-detecting animals such as echolocating bats.

This study also serves as a prelude to future work that will provide a detailed mapping of the brain regions associated with ultrasound detection and provide physiological support to the suggestion that the utricle is the site for ultrasound transduction.

METHODS

Experimental animals and setup

Data were collected from 29 American shad (A. sapidissima) ranging in body length (snout to base of caudal fin) from 10 to 23 cm. Fish were obtained from a fish farm and fed daily until they were used in the studies. They were kept in round communal freshwater tanks (fiberglass, 0.5 cm thickness, 123 cm diameter, 73 cm deep) and on a 14:10-h light-dark cycle. All work was approved by and under the...
Stimulation

Sounds in the sonic frequency range were produced with an underwater loudspeaker (UW 30, University Sound), whereas ultrasonic sounds were produced through an ultrasonic transducer (ITC 1042) that can be used as a sound transmitter or receiver. The sonic loudspeaker was mounted at the bottom of the lower tube but was isolated mechanically from it with a rubber gasket so that the motion of the speaker could not be directly imparted into the walls of the tank. The ultrasonic hydrophone was hung directly above the sonic speaker and did not touch the walls of the tank (Fig. 1A).

Sonic and ultrasonic signals were generated using a computer-guided AD/DA converter (RP2, Tucker-Davis Technologies (TDT)). The signals were DC filtered (at 0.1 Hz) by a programmable attenuator (PA5, TDT) and fed into a power amplifier (Havel). The amplified signal was then fed into an oscilloscope and into the sonic or ultrasonic transducer depending on the frequency being used. The sonic speaker was used for 100-, 200-, 300-, 500-, 800-, 1,000-, 2,000-, 3,000-, 5,000-, and 8,000-Hz pure-tone signals. The ultrasound hydrophone was used for 10- to 90-kHz signals in 10-kHz intervals. The stimulus amplitude was set at 40 dB above the hearing threshold of the fish as determined in an earlier behavioral study (Mann et al. 1997) (Fig. 2).

Stimulus duration was adjusted to 300 or 500 ms for sonic stimulation and 100 or 300 ms for ultrasound stimulation. Depending on the frequency, signals had a 2- or 50-ms rise and fall time. All sonic and ultrasonic signals were calibrated with an ITC transducer. The calibration hydrophone output was amplified (VBF-7, Stewart), sampled at a rate of 1 MHz (IoTech Wavebook 512), and stored on a PC. Stimulus signal spectra and amplitude at the position of the fish were analyzed with Matlab (Mathworks) and Igor Pro (Fig. 3).
In general, we used search stimuli across frequencies of 20–90 kHz. When a unit was found, it was tested with all stimulus signals (sonic and ultrasonic) in random order. In several cases, sonic search stimuli were used when ultrasonic search stimuli failed. Only in two cases were sonic units tested with all frequencies for control purposes.

Histology

In a few cases, the recording sites were marked with either an electrolytic lesion or the neurotracer Neurobiotin. For neurotracer applications, Neurobiotin-filled electrodes were given a positive DC current (≥7 μA) for three 2-min periods separated by a 1-min period of negative current to avoid clogging the electrode tip. After the recordings were made, fish were deeply anesthetized with buffered MS-222 and perfused intracardially with freshwater teleost Ringer solution followed by 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed and cut at 30 μm in a transverse plane parallel to the electrode penetrations. The Neurobiotin was labeled with 3,3'-diaminobenzidine, and the sections were counterstained with neutral red and analyzed under a microscope. Electrolytic lesions were made with a 12-V power supply hooked to the electrode. The polarity of the current was altered each minute for a total duration of 6 min. We were not able to measure the current flow at the tip of the electrode. Electrolytic lesion slices were counterstained with neutral red. Slices with lesions were digitized and stored on a PC.

RESULTS

The primary goal of this study was to determine the general nature of ultrasound processing in the brain stem and midbrain of American shad and provide a baseline for future and more quantitative studies of the site(s) of ultrasound processing. Accordingly, we only provide a qualitative description for the recording site of most units. Later studies will provide a more detailed quantitative analysis of sites of ultrasound processing in these regions.
Types of units and recording depth

Sixty-two ultrasound-sensitive units were recorded in the 29 experiments. Ultrasound-sensitive units could be found between recording depths of 110 and 4,510 μm. In six cases, either Neurobiotin tracer \((n = 3)\) or electrolytic lesions \((n = 3)\) were identified. We did not correlate the recording depth of units to brain regions because this was not a quantitative study. At the same time, we did find that tracer applications were located within the granule cell layer of the vestibulolateral lobe of the cerebellum and the medial portion of the rostral region of the eminentia granularis (EG) (Fig. 5). Electrolytic lesions were found in nuclei of the afferent acoustic pathway, the secondary octaval population (SO), and the descending octaval nucleus (DON).

Twelve of the 62 units \((19\%)\) showed inhibitory characteristics in their response to the stimulus (for example, see Fig. 6A), whereas the rest showed excitatory characteristics in response (for example, Figs. 6D and 7). Eighty percent of the units showed phasic responses to either sound onset or offset or to both, whereas 12 units \((\text{inhibitory and excitatory})\) showed changes in their response characteristic as stimulus frequency increased (Fig. 6). Inhibitory units were recorded at all depths.

Six ultrasound-sensitive units had properties that resemble the precise timing and the sharply tuned response characteristics of constant latency units first described in the columnar area of the ventral division of the ventral nucleus of the lateral lemniscus in bats (VNLLv) (Covey and Casseday 1991) (Fig. 7). These units were found dorsally at depths between 260 and 2,800 μm.

Eighty-eight percent of the ultrasound-detecting units responded to frequencies above 60 kHz, and of these units, 90% had best frequency responses between 70 and 90 kHz (Fig. 8). No ultrasound-sensitive units responded to the whole range of test frequencies \((20–90 \text{ kHz})\). Although some units showed a broader frequency range than others (e.g., Fig. 7), there were too few units to attempt to provide a classification scheme at this time.

The overall distribution of onset latency showed a continuum from 2 to 44 ms with a small peak at 14 ms and an average latency of 17.5 ms (Fig. 9A). The latencies showed no statistically significant correlation with recording depth (Fig. 9B) or stimulus frequency (Fig. 9C).

Phase coupling was calculated for ultrasound-detecting units using the circular statistic of Goldberg and Brown (1969). None of the single-unit responses showed any trace of phase coupling to ultrasound (Fig. 10). We did not attempt to determine whether the few lower ultrasound frequency units \((<50 \text{ kHz})\) or sonic range units show phase coupling.

Ultrasound responding units showed a wide diversity in spontaneous activity rates, ranging from 0.08 to 115 spikes/s with an average of 15.6 spikes/s. However, there was no clear correlation between spontaneous activity and recording depth (Fig. 11). In addition to the ultrasonic units, a few sonic frequency units were also encountered, all of which responded below 1 kHz. As controls, we recorded and analyzed two of these units, both of which were found between 1,000 and 1,200 μm deep. There were too few units to provide any statistical analysis of their characteristics.
DISCUSSION

Although the stimuli used here only resemble the frequency and amplitude components of the natural echolocation signal of odontocetes (but not the signal duration or frequency characteristics), they allow for a "spectral analysis" of the neuronal pathway of the American shad. Moreover, despite the experiments being done in a small tank, they provide important insight into the nature of neuronal responses to sounds in American shad that may be produced by potential predators (Au 1993; Domenici et al. 2000; Houser et al. 1999).

FIG. 7. Example of a constant latency unit. These units were characterized by short constant latencies (2–4 ms) to stimulus onset and a broad response area in the ultrasound range. A: 50-kHz Raster plot; B: 70-kHz Raster plot; C: 90-kHz Raster plot; D: shape of action potentials after spike sorting (single unit). ■ below C; stimulus duration.

FIG. 8. Distribution of ultrasound-sensitive unit responses and their best frequencies. Light-gray bars show the total number of responses of all units to a given sonic or ultrasonic stimulus independent of response strength. Dark-gray bars show the total number of strongest responses of all units at the given stimulus frequency. Note that every unit responded at several frequencies (a) and some units showed equally strong responses at different stimulus frequencies (b). Thus the total number of strongest responses (n = 74) is bigger than the number of units recorded (n = 62) and most inhibitory units were included (c).

FIG. 9. Latencies to stimulus onset of all units (excitatory and inhibitory). A and B: averaged latency at given recording depths; C: onset latency at given stimulus frequencies. Note that responses were calculated only for those responses that occurred at stimulus onset.
Neuronal responses to ultrasound

American shad show behavioral responses to ultrasound at frequencies up to 180 kHz. Sensitivity at ultrasonic frequencies is relatively poor (<140 dB SPL at best frequencies between 50 and 120 kHz), whereas the sensitivity to sonic range signals <1 kHz is considerably better (~120 dB) (Mann et al. 1997) (Fig. 2). Nevertheless, the overall hearing range measured with behavioral methods extends from <1 up to 180 kHz, a bandwidth found in no other animal (Fay 1988; Hoy 1992). Most importantly, an analysis of signal levels produced by echolocating dolphins compared with ultrasonic sensitivity shows that American shad should be able to detect dolphins at distances >100 m (Au 1993; Mann et al. 1997).

Our data show that no single neuron responds to this very broad frequency range of hearing. In addition, we found few units that responded to stimulus frequencies between 20 and 40 kHz despite hearing threshold measurements showing that American shad can detect these sounds (Mann et al. 1997, 2001). It is of considerable interest that the electrophysiological results coincide with behavioral avoidance data (Plachta and Popper 2003) in showing no responses below 40 kHz. Thus although American shad can detect signals from 20 to 40 kHz as determined by measuring hearing sensitivity (Mann et al. 1997, 2001), the neuronal sensitivity to these frequencies might not be represented in the region of the brain in which we recorded.

Not only were there few units responding to frequencies between 20 and 40 kHz, there were also very few individual units that responded to both ultrasonic and sonic frequencies. While using a stimulus amplitude level that was 40 dB above the hearing threshold, it seems remarkable that only higher ultrasound frequencies are strongly represented in areas of the CNS that we recorded from in American shad. Of course, it is possible that sonic and ultrasonic regions of the brain are segregated and that the locations used in our study were primarily for ultrasonic responses.

Because so many high-frequency ultrasound-sensitive units were driven by our pure-tone stimulus, it appears reasonable that the same distribution of neuronal frequency selectivity might also be found on the level of the primary afferent neurons. Thus it appears that the signals used here contained a substantial portion of the information the fish needs for signal analysis. A possible model for such a system might be a highly specialized inner ear with distinct receptive areas and innervation for sonic sound and for ultrasound with the sensitivity for the ultrasound shifted toward frequencies above 40 kHz. This would correspond with recent behavioral work on freely swimming American shad in which only frequencies from 70 to 110 kHz triggered the strongest evasive responses (Plachta and Popper 2003).

Pathways for sound processing?

In bats, the processing of ultrasound information is dominant from the periphery to the inferior colliculus (IC), but as in moths, no separate pathways for sonic and ultrasonic signals are known (Haplea et al. 1994). However, unlike in moths, the afferent acoustic pathway in bats is functionally separated into two subsystems: the monaural and the binaural systems with the latter receiving input from both ears (Boyan and Miller 1991; Covey et al. 1991).

Our results suggest that American shad may use two separate pathways. Very few units recorded in this study showed sensitivity to both ultrasonic and sonic stimuli even though these units were found in nuclei belonging to the acoustic pathway. Furthermore, the distribution of units responding to the ultrasound and sonic ranges appear to be separated by a large frequency gap that has only few responsive units, and these units never had their best frequencies between 1 and 50 kHz. These findings, along with results showing that sonic white noise masking had no affect on avoidance response of American shad to ultrasound stimuli (Plachta and Popper 2003), provide initial evidence that the processing of ultrasonic signals in American shad might be independent from the processing of sonic range signals (O’Connell 1955).

In the afferent pathway of bats, units sharpen and become more precise in response to pure-tone stimulation (e.g., constant latency neurons in the VNLLv) (Covey and Casseday 1991). These responses serve as input to IC neurons for confluence of different sound features such as duration, frequency, intensity, direction, spatial pattern, and auditory scene analysis (Casseday and Covey 1996; Covey 2001). Our recordings demonstrated units that resemble the response properties of
units typically found in the VNLLv of bats, i.e., constant latency units (see Fig. 7).

Peripheral site of ultrasound detection

The responses obtained from auditory nuclei of the brain strongly support the hypothesis that the responses to ultrasound are mediated by the ear. Our morphological data show that the recording sites are among the granula cells of the vestibulolateral lobe of the cerebellum in the vicinity of the medial region of the rostral eminentia granularia, an area that is known to receive input from the afferent of eighth nerve in fishes (Bass 1982; Bell 1981; McCormick 1997; Song and Northcutt 1991). Moreover, in one or two of our Neurobiotin-labeling cases, lightly labeled afferent fibers can be observed and traced from the eminentia granularia lesion site back to the fascicle at the root entering the eighth nerve. All of these observations support the suggestion that the inner ear is the ultrasound receptor, and the specific site may be in one of the three sensory epithelia in the utricle of the ear (Higgs et al. 2004; Mann et al. 1997).

Our results do not preclude there being an “extra ear” in American shad, but in comparison with close relatives that do not detect ultrasound (e.g., bay anchovy, sardine), it is hard to imagine that a totally new system for ultrasound detection would have evolved in one small group of clupeids and not in the whole group. Still, only tracer studies will provide the evidence needed to tell which end organ functions as an ultrasonic receiver.

Response characteristics of ultrasound-sensitive units

Ultrasound-sensitive units showed considerable variety in their response characteristics. Although some units showed unique response patterns over a broad frequency range, others changed their response characteristics with every 10-kHz increase in test frequency (Fig. 6).

One might have assumed that ultrasound detection by American shad is nothing more than a primitive ultrasonic detector and that ultrasound detection evolved for the determination of the presence of a sound of biological importance to the animal. Our neuronal data, however, lead us to a quite different suggestion. We found a group of feature-rich ultrasound-sensitive units that responded in a wide range of response patterns. Some of these responses are understood, whereas others need to be explored further (e.g., onset response switches to offset response and back again if the stimulus frequency is increased). Also, the inhibitory responses provide at least indirect evidence for complex signal processing. Thus rather than being only a simple detector of the presence of ultrasound, our data lead to the suggestion that there is some sophistication in the ability of American shad to process such signals.

The response characteristics of the ultrasound-sensitive units are broadly variable at different recording depths, and thus there is no clear feature that could be assigned to a certain recording depth or nuclei at this time. The information on location and distribution of ultrasound-sensitive units combined with an enhanced stimulus method (variable direction, amplitude, amplitude-FM, and pulsed stimuli) will allow for far more focused investigations on the function of the different processing stages.

It is not surprising that we did not find phase coupling to the test signals in any of the ultrasound-sensitive units. Nevertheless, some units showed very precise timing relative to the onset of the stimulus. How this timing is achieved will be a subject of further investigations, particularly in the units that show constant latency to stimulus onset. Units such as these were first described in detail in the afferent pathway of bats and were assumed to play a major role in binaural calculation of directional information (Covey 2001). Interestingly, the presence of directional evasive behavior by American shad along with constant latency units in the brain suggests a relationship between these units and the motor system that may mediate a directional escape response. A new series of experiments using a setup capable of directional stimulation would be necessary to find out how American shad handle the remarkable problem of processing temporal information from a signal front passing the two ears in <3 ms.

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