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USING CLICK-EVOKED AUDITORY BRAINSTEM RESPONSE WAVEFORMS TO MEASURE HEARING LOSS IN THE BOTTLENOSE DOLPHIN (*TURSIOPS TRUNCATUS*)

by

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Developing an improved methodology for efficient and informative hearing assessment in marine mammals would prove insightful in expanding current knowledge regarding marine mammal hearing sensitivity, particularly in studying the effects of anthropogenic noise. This study examines the click-evoked auditory brainstem response in relation to upper frequency limit of hearing in the bottlenose dolphin (Tursiops truncatus) as a proxy for hearing capacity.

ACKNOWLEDGMENTS

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INTRODUCTION

Basic Mechanisms of Audition in Odontocetes

Odontocetes are a suborder of cetacea that includes the toothed whales (dolphins and porpoises). Given that sound travels about five times more efficiently in water than light, cetaceans have evolved to utilize audition as their primary sensory modality for activities such as navigation, foraging, predator avoidance, and (likely) social communication. Though not unique to odontocetes, the evolution of echolocation, or biosonar, enabled odontocetes to exploit information about their environment at greater distances than is feasible through vision while underwater. The evolution of echolocation in odontocetes was accompanied by an increase in hearing frequency range, which has a bandwidth roughly comparable to the usable frequency bandwidth of echolocation frequencies. Odontocete echolocation abilities currently surpass any man-made systems and allow for underwater object identification and discrimination from hundreds of meters away in addition to identification of buried objects. For this reason, interest in the odontocete auditory system has a significant history of scientific investigation.

Norris was the first investigator to hypothesize that sound reception is associated with the odontocete mandible as opposed to the external auditory meatus as in terrestrial mammals. More specifically, he proposed that sound enters the odontocete auditory system via an oval region of fatty tissue (the "acoustic window") that covers a thin, translucent area of the lower jaw termed the pan (Norris, 1964; Norris, 1968), which is sometimes incorrectly referred to as the pan bone. From the pan region of the mandible, sound propagates to fatty bodies filling the mandibular canal ("acoustic fats"), providing a low-impedance propagation pathway. The acoustic fats are in direct connection with the tympanic bulla, the structural housing for the odontocete middle ear. However, unlike other marine mammals (baleen whales, sea lions, and seals) and terrestrial

mammals, the auditory bullae are highly dense and anatomically disparate from the bones of the skull, allowing for improved translational bone conduction of high frequency stimuli (McCormick, Weaver, Ridgway, & Palin, 1980). Norris' "jaw hearing" hypothesis was later confirmed by a variety of studies utilizing behavioral and electrophysiological methodologies (Bullock et al., 1968; Renaud & Popper, 1975; Brill, Sevenich, Sullivan, Sustman, & Witt, 1988; Popov & Supin, 1990a; Popov, Supin, Klishin, Tarakanov, & Plentenko, 2008). It is also important to note that Ketten (1994) identified a novel fat channel associated with sound reception in a few odontocete species (including the Atlantic bottlenose dolphin, *Tursiops truncatus*) using magnetic resonance imaging (MRI). This additional fat channel is lateral to the tympano-periotic complex and is thought to provide a more efficient sound pathway for low frequencies as compared to the jaw pathway for high frequencies utilized in echolocation (Ketten, 1994).

Unlike terrestrial mammals, McCormick, Wever, and Palin (1970) identified no functional use of the external auditory meatus, tympanic conus, tympanic membrane, and tympanic ligament in odontocetes. The tympanic membrane is not in direction connection to the malleus and is attached by only one ligament, thought to function in suspension (McCormick et al., 1970). After reaching the tympanic bulla, sound is amplified by the middle ear ossicles, with the incus and stapes articulations providing the most significant amplification (at least for high frequencies) (McCormick et al., 1970). Even though there is a minimal impedance mismatch between the odontocete's aquatic environment and cochlear fluids, studies modeling the tympano-periotic complex suggest a function in velocity amplification utilizing a lever action (Nummela, Reuter, Hemila, Holmberg, & Paukku, 1999). Furthermore, Ketten (1994) described the odontocete ossicles as rigid and calicified compared to terrestrial mammals with

interossicular joints stiffened by ligaments and a fibrous sheath. This extremely stiff middle ear system is thought to be an adaptation for ultra-high frequency hearing (Ketten, 1994; Ketten, 1997). Additionally, odontocete middle ear resonance is determined by cavity air volume, which can change as a result of varying hydrostatic pressure associated with frequent deep-water dives (Ketten, 1994). The middle ear cavity is highly innervated with trigeminal nerve fibers and is associated with a distensible tissue, the corpus cavernosum, which potentially allows for active adjustment of pressurization while diving (Ketten, 1994).

Following amplification via the ossicular chain, sound reaches the oval window of the cochlea within the periotic bulla where it is hypothesized that relative motion of the stapes footplate and the otic capsule allows for cochlear fluid (more specifically, basilar membrane) displacement and fine-frequency tuning (McCormick et al., 1970; McCormick et al., 1980). Similar to the mechanisms described in terrestrial mammals, frequency resolution along the basilar membrane is based upon stiffness and thickness, with the basal end of the membrane being most sensitive to high frequencies (stiff and thin) and the apical end being most sensitive to low frequencies (flaccid and thick). Additional stiffening of the basal turns (i.e. high frequency regions) via bony outer laminar supports is also thought to contribute to ultrasonic hearing (Ketten, 1994). Little is known regarding sensory transduction at the cochlear hair cells (HCs) in odontocetes, but cellular anatomy is similar to that of terrestrial mammals with a few key differences. Ketten (1997) noted a high density of afferent innervations (up to 2,900 ganglion cells) and a high density of HCs (up to 100 inner hair cells (IHCs)/mm; up to 300 outer hair cells (OHCs)/mm). In some odontocete species, there were up to three times as many ganglion cells per IHC as compared to humans (Ketten, 1997).

In addition to low-pass filtering at the auditory nerve, auditory areas of the brainstem, and auditory areas of the cortex, reintegration of ipsilateral information throughout the odontocete auditory nervous system allows for sound localization (Mooney, Yamoto, & Branstetter, 2012). More specifically, the "core loop" (recurrent circuit), thought to be primarily responsible for localization by echolocation, receives input from the auditory cortex (anterior cingulate cortex) and includes the following neural structures: elliptic nucleus -> medial tegmental tract -> inferior olive -> cerebellar cortex -> posterior interposed nucleus -> elliptic nucleus (Oelschläger, 2008). An additional loop thought to be significantly involved in echolocation function receives input from the neocortical auditory and motor centers and includes the parafloccular cortex -> posterior interposed nucleus -> elliptic nucleus (Oelschläger, 2008). The aforementioned loops function in sensory input processing and integration from sources including the ascending auditory pathway and descending projections from auditory neocortical areas (Oelschläger, 2008).

For sound localization in the horizontal plane, interaural level differences (ILDs) and interaural time differences (ITDs) are used (Mooney et al., 2012). External ILDs are reduced in an aquatic environment due to density similarities between water and the odontocete head. However, internal ILDs are created by internal anatomical structures of varying density, similar to other auditory predators such as the barn owl (*Tyto alba*) (Knudsen, 1981). Albuminous foam (air sinuses, lipids, and vascularization), cranial air sacs, and mandibular fats partially isolate one ear from the other, creating ILDs as large as 20 dB for both narrow and broadband stimuli and ILD sensitivity < 1 dB (Ketten, 1992; Supin & Popov, 1993; Moore, Pawloski, & Dankiewicz, 1995). The use of ITDs is also inhibited by the aquatic environment given that sound travels about five times faster in water than in air. However, ITD sensitivity as small as seven

microseconds has been measured by Moore et al. (1995). Spectral cues (specifically, interaural spectral differences) are also thought to be a significant tool in aquatic sound localization in the horizontal plane (Supin & Popov, 1993).

Auditory-Evoked Potentials in Odontocete Hearing Sensitivity: A Brief History

Auditory evoked potentials (AEPs) are small changes in voltage representing neural synchrony within the auditory nervous system in response to acoustic stimuli. The auditory brainstem response (ABR) is an AEP generated specifically from the auditory nerve and within the auditory brainstem. In odontocetes, the ABR is a robust and replicable response quantified by amplitude and latency values of seven waveforms, all occurring within about 6 ms of the stimulus onset. As stimulus intensity decreases, the ABR waveform amplitudes decrease and latency values increase. These trends are also observed as stimulus repetition rate increases.

Although AEPs allow non-invasive (far-field) measurement of hearing sensitivity, the earliest studies of AEPs in odontocetes utilized invasive, direct (near)-field recordings requiring anesthesia (Bullock et al., 1968; Seeley, Flanigan, & Ridgway, 1976; Ridgway, 1980; Ridgway, 1981; Ridgway, 1983; Popov & Supin, 1985). Direct (near)-field recordings involve electrode placement at or near the response neural generator sites, whereas far-field recordings involve electrode placement away from the response neural generator sites with little to no corresponding effect(s) upon amplitude and/or latency. The studies listed above provided the foundational knowledge required for the effective measurement of far-field recordings conducted today. Bullock et al. (1968) performed the first comprehensive study of AEP recordings in anesthetized odontocetes, with recordings from 29 individuals representing 4 species (*Stenella caeruleo-alba, Stenella attenuata*, *Steno bredanesis*, and *Tursiops truncatus gilli*). Short duration (0.3 – 10 ms) tone pips (5 – 150 kHz) with variable rise-fall times (≥ 0.1 ms) were presented via air, water, and

direct (tactile) stimulation against the skin of the head (Bullock et al., 1968). Tungsten and

stainless steel electrodes were used in direct-field recordings from the inferior colliculi,

medullary auditory centers, and the medial geniculate bodies (Bullock et al., 1968). The most

reliable response was recorded from the inferior colliculus (Bullock et al., 1968). Additional

information regarding waveform characteristics, temporal resolution, electrode placement,

frequency tuning, masking using background noise, and utilizing pure tones versus modulated

stimuli was presented (Bullock et al., 1968). Direct-field recordings also allowed identification

of potential neuroanatomical correlates of the evoked response, such as those described in

humans by Spehlmann (1985): wave I from the auditory nerve, wave II from the cochlear

nucleus and trapezoid body, wave III from the superior olivary complex, wave IV from the

lateral lemniscus, wave V from the inferior colliculus, and waves VI and VII from the medial

geniculate body of the thalamus. However, these neuroanatomical correlates have not been

precisely defined in odontocetes species. The Marine Mammal Protection Act of 1972

discontinued invasive studies of this nature in the United States, but similar work conducted by

Soviet scientists allowed for identification of cortical auditory response areas and variance of

AEP response onset and offset based upon stimulus frequency and duration (Ladygina & Supin,

1970; Popov & Supin, 1976; Ladygina & Supin, 1977; Popov & Supin, 1978).

The first study utilizing minimally invasive methodology (i.e. needle electrodes placed 2

– 3 mm into the skin) in unanesthetized odnotocetes was by Popov and Supin (1990a). Popov

and Supin (1990a) presented a comprehensive study measuring ABR amplitude as a function of

electrode placement and stimuli characteristics. The largest ABR amplitudes were recorded when

the active electrode was placed about 6 cm behind the dolphin's blow hole (Popov & Supin,

1990a). The lowest thresholds were measured utilizing tone bursts at 80 kHz (Popov & Supin, 1990a).

Although the use of electrophysiological methods such as the ABR eliminated a requirement for access to captive individuals and extensive training time for behavioral measures of hearing sensitivity, this methodology is not without fault for frequency-specific assessment (as described in Au & Hastings, 2008). However, with the development of auditory steady-state response methodology (ASSR; also termed the envelope-following response or EFR), frequency specificity was made possible, further enabling comparisons between behavioral and electrophysiological data obtained under varying stimuli levels and durations (Au & Hastings, 2008). A common ASSR methodology used in odontocetes for frequency specific tests of hearing sensitivity involves presentation of sinusoidal amplitude-modulated (SAM) tones modulated at a rapid rate and with a specified degree of amplitude modulation depth (typically 100%). The recorded neurophysiologic response follows the "envelope" of the amplitude modulated carrier signal such that the ASSR is detected as a voltage peak at the modulation rate (frequency). In other words, the auditory neurons are responding to the carrier tone but firing at the modulation rate (Finneran, London, & Houser, 2007). Optimal presentation rates can be found by the determining the modulation rate transfer function, which is defined as the relationship between the amplitude of the evoked response and the modulation frequency for a fixed carrier frequency. In the bottlenose dolphin, peak amplitudes are recorded when using modulation frequencies ranging from 550 – 600 Hz, 1000 – 2000 Hz, and 1400 – 1700 Hz (high frequency carrier signals only) despite the presence of high frequency hearing loss (Dolphin, Au, Nachtigall, & Pawloski, 1995; Supin & Popov, 1995; Finneran et al., 2007). The ASSR is now commonly used in odontocete hearing sensitivity assessments and has shown good agreement

with behavioral measures of hearing sensitivity, although it typically underestimates behavioral sensitivity to some degree (Nachtigall, Supin, Pawloski, & Au, 2004; Houser & Finneran 2006b; Finneran et al., 2008; Houser, Gomez-Rubio, & Finneran, 2008). Additionally, more specific comparisons, such as the examples listed below, have established good agreement between electrophysiological (ASSR) and behavioral thresholds using a jawphone transducer placed on the pan region of the mandible: underwater ASSR thresholds versus underwater behavioral thresholds (Houser & Finneran, 2006a); aerial ASSR thresholds versus underwater behavioral thresholds (Finneran & Houser, 2006); and aerial ASSR thresholds and behavioral thresholds collected simultaneously (Schlundt, Dear, Green, Houser, & Finneran, 2007).

Population Level Hearing Sensitivity: Atlantic Bottlenose Dolphin (*Tursiops truncatus*) and Pacific Bottlenose Dolphin (*Tursiops truncatus gilli*)

Ridgway and Carder (1997) were the first to describe hearing sensitivity and trends in multiple Atlantic bottlenose dolphin (*Tursiops truncatus*) using behavioral methodologies in 8 dolphins (4 males and 4 females) ranging in age from 7 – 35 years. Incidence of hearing loss was correlated with age and gender. Older individuals were more likely to have high frequency hearing loss, or presbycusis (progressive hearing loss with age typically beginning in the high frequencies), and males had higher incidence and severity of high frequency hearing loss (Ridgway & Carder, 1997). These trends are consistent with those experienced by terrestrial mammals, including humans.

Houser and Finneran (2006b) presented similar findings using ASSR methodology in the first population-level assessment of bottlenose dolphins; specifically, utilizing 42 Atlantic bottlenose dolphins (28 males and 14 females) ranging in age from 4 – 47 years. Presbycusis was experienced in the high frequencies first (> 100 kHz) and tended to occur at ages within the mid-

twenties (20 – 30 years of age), although some older animals still had full range of hearing. A statistically significant finding was that males began to lose their high frequency hearing before females (Houser & Finneran, 2006b). They also found abnormal patterns of hearing sensitivity in two dolphins that were thought to be related (father and son), suggesting a potential genetic component to hearing loss in dolphins analogous to terrestrial mammals (Houser & Finneran, 2006b).

A similar study by Houser et al. (2008) quantified hearing sensitivity using ASSR methodology in 13 Pacific bottlenose dolphins (5 males, 8 females) ranging in age from 1.5 – 18 years (2008). High frequency (> 60 – 80 kHz) presbycusis was documented in two animals with estimated ages of 17 and 18 years. One dolphin presented with an abnormal hearing sensitivity configuration: notches at 30 and 100 kHz (Houser et al., 2008). Interestingly, in comparison to the Atlantic bottlenose dolphin mean audiogram provided in Houser and Finneran (2006b), the Pacific bottlenose dolphin mean audiogram was significantly more sensitive at 40, 60, 80, 100, and 115 kHz (Houser et al., 2008). Although the exact reason cannot be identified, genetic rather than methodological and/or noise exposure differences is thought to be a possible factor (Houser et al., 2008).

Justification for the Current Study: Proposed Additions to Current AEP Methodology

Due to the negative impacts of anthropogenic noise upon marine mammals (see Miller, Abbas, & Brown, 2000 and Holt, Noren, Veirs, Emmons, & Veirs, 2009), the National Research Council (NRC) has repeatedly documented the need for additional research required to better understand marine mammal hearing sensitivities and the physiological impact of sound on marine mammals (e.g. temporary threshold shift) (NRC, 1994; NRC, 2000; NRC, 2003; NRC, 2005). Recommendations such as establishing baseline hearing sensitivities in greater numbers

of species and individuals representing these species have been outlined, requiring AEP equipment that is hardy and portable (such as the system described in Finneran, 2009) and methodologies that are easily programmable for automaticity and time-efficiency, particularly in the case of field testing (i.e. stranded animals).

This study hypothesizes that conducting ABR testing using a suprathreshold click stimulus to estimate the upper frequency limit of hearing in *Tursiops truncatus* could prove a more expedited methodology compared to the SAM tone-evoked ASSR, which is now commonly used to study odontocete hearing. The results of this study have potential applications to marine mammals both in the wild (i.e. increasing the number of individuals representing a species and the number of species for which hearing sensitivity data exists, therefore expanding current knowledge of inter- and intraspecies variation) and under human care (i.e. more routine assessment of hearing sensitivity). For Navy dolphins specifically (the subjects of this study), high frequency hearing for use in echolocation is of the utmost importance, enabling the animal to perform his/her tasks (e.g. underwater mine hunting or swimmer detection and interdiction). Therefore, the results of routine hearing assessment play a vital role in determining task assignment and in monitoring the auditory system health of these acoustically-dependent animals. For these reasons, a more efficient testing methodology would prove clinically useful by reducing the logistical burden associated with testing and increasing the frequency at which testing occurs.

MATERIALS AND METHODS

Subjects

Study subjects were Atlantic bottlenose dolphins (*Tursiops truncatus*) in care of the

United States Navy Marine Mammal Program at the Space and Naval Warfare Systems Center

Pacific located in San Diego, California (SSC Pacific). Subjects included two dolphins with

normal hearing (1 male, 1 female) and four dolphins with high frequency hearing loss (3 males, 1

female) ranging in age from 13 – 49 years. Hearing loss was defined in this study as an upper

frequency limit of hearing ≤ 100 kHz. All protocols were approved by the Institutional Animal

Care and Use Committee of the Biosciences Division, SSC Pacific, and followed all applicable

U.S. Department of Defense guidelines for the care of laboratory animals.

Stimulus Presentation and Evoked Response Recording

All subjects were tested in open-ocean netted enclosures in San Diego Bay (SD Bay)

positioned submerged on a biteplate with their dorsal surface above the waterline, allowing for

ease of respiration throughout the test sessions. Acoustic stimuli were presented to the subject

utilizing a jawphone transducer (piezoelectric sound projector [Reson TC 4013] embedded in a

V-1065 silicon rubber suction cup) placed on the pan region of the left mandible (Moore et al.,

1995; Brill, Moore, Helweg, & Dankiewicz, 2001) (Figure 1). The jawphone transducer was

calibrated with the same stimuli used for the study (SAM tones and high-intensity clicks) and

was calibrated at a distance of 15 cm from the transducer. This distance was used as it

corresponds to the distance between the attachment point of the transducer on the lower jaw and

the auditory bulla. The dolphins were provided positive reinforcement in the form of fish for

their voluntary participation in the study.

SAM Tone-Evoked ASSR

SAM tones generated by a portable auditory-evoked potentials system (EVREST, detailed in Finneran, 2008; Finneran, 2009; and Finneran, Houser, Mase-Gurthrie, Ewing, & Lingenfelser, 2009) were used to evoke an ASSR. The SAM tones consisted of one of seven carrier frequencies spaced at half octave steps from 20 – 160 kHz. Each SAM tone was 100% amplitude modulated at a rate of 1 kHz; this modulation depth and rate has been shown to be optimal for evoking a robust ASSR in the Atlantic bottlenose dolphin (Dolphin et al., 1995; Supin & Popov, 1995; Supin & Popov, 2000). All SAM tone stimuli were generated with a 1 ms rise/fall time and were 22 ms in duration.

Click-Evoked ABR

Click stimuli of various durations (5, 50, and 100 μs) were generated by transmitting a 1 V rectangular wave to the jawphone transducer also using the EVREST system (see above). The transmitted click had a peak-peak equivalent sound pressure level (pp_eSPL) of 122 dB re 1 μPa (hereafter denoted as "dB SPL"). Clicks were presented to the dolphins at a rate of 46.8 clicks/s and the polarity of the click was alternated on each presentation to cancel out any potential artifacts from the stimulus.

Evoked Response Recording

The ASSR was measured utilizing gold-cup electrodes (Grass FH-E6G series) embedded in 25 mm diameter silicon suction cups coupled to the skin using conductive paste. Electrodes were placed by the investigator (KGR) immediately prior to each test session in the following montage: noninverting (+) electrode at ~ 4" posterior to the inferior margin of the blowhole and ~ 1" contralateral of the ear being tested; common (ground) electrode on the subject's back ~ 3" anterior from the dorsal fin; and inverting (-) electrode placed on the subject's back at the

halfway point between the noninverting and ground electrodes (Popov & Supin, 1990a) (Figures 1 and 2). Electrode signals were differentially amplified (100,000 gain), filtered (300 Hz – 3 kHz), and digitized at 1 MHz. The signal rejection level (i.e. artifact rejection) was set at the beginning of each session based on the background electrophysiological noise observed prior to the beginning of sample collection.

A magnitude-squared coherence (MSC) test was applied after 256 epochs to determine if the amplitude of the evoked response at the modulation frequency was significantly greater than measurement noise (Dobie & Wilson, 1989; Dobie, 1993; Dobie & Wilson, 1996). The test was repeated utilizing the cumulative number of epochs recorded every 256 epochs until the signal was detected or until a maximum of 1024 epochs was recorded. Utilizing the ASSR that corresponded to full amplitude modulation of the stimulus (i.e. ignoring the rise/fall component), the MSC was calculated by dividing the total number of epochs obtained for each frequency/stimulus pairing into 16 subaverages. The MSC_{crit} for each test was obtained from Amos and Koopmans (1963) and Brillinger (1978) assuming an alpha = 0.01. Signals with a $MSC > MSC_{crit}$ were considered statistically different from noise, and thus, detected responses.

Initial testing was performed to determine an estimate of hearing threshold at each test frequency and additional frequencies were tested to determine the upper frequency limit of hearing: 90 kHz, 100 kHz, 120 kHz, and 130 kHz. An automated modified staircase technique was used to adjust the stimulus intensity and record responses sufficient for threshold estimation. Data collection began with a stimulus level of 110 dB SPL (exception: testing at 160 kHz which began at 120 dB SPL). If a signal was detected, the SPL was reduced for the subsequent test. The initial change in SPL for subsequent tests began at 30 dB (exception: testing at 160 kHz which began at 10 dB). If the ASSR was not detected, the SPL was increased on subsequent tests until

it was once again detected. The change in the SPL on subsequent tests was adjusted upon each reversal; the step size was decremented by 0.45 of the prior step size when reversing from a non-detection to a detection, and was decremented by 0.40 of the prior step size when reversing from a detection to a non-detection. The testing concluded when the step size was ≤ 3 dB and the threshold was calculated as the difference between the lowest stimulus SPL producing a detectable ASSR and the highest stimulus SPL at which no ASSR was detected. Threshold testing was terminated if no detections were obtained with stimulus SPL ≥ 120 dB SPL. The upper frequency limit of hearing was defined as the frequency at which threshold was equal to 120 dB re: 1 μPa. It was determined by linearly interpolating between two frequencies with thresholds above and below the 120 dB criterion.

An ASSR-derived input/output (I/O) function was determined for each animal at each frequency for which a threshold < 120 dB SPL could be determined. To create the I/O function, the amplitude of the ASSR was first determined for a SAM tone stimulus of 40 dB sensation level (SL), i.e. 40 dB above the initially determined threshold. (When thresholds were determined but stimulation at 40 dB SL was not possible, stimulation began at the highest stimulus level producible by the transducer without producing stimulus artifacts). The stimulus SPL was decreased in 5 dB increments until 10 dB below SAM threshold and 1024 epochs were recorded at each stimulus level tested. The amplitude of the evoked response spectra at the modulation rate, determined from the average of the 1024 epochs, was subsequently plotted for each stimulus level presentation to determine the I/O function. Following visual inspection of the data confirming a break point, a segmented regression analysis was used to determine if a break-point truly existed in the I/O function (i.e. a notable change in the slope of the I/O function within the range of tested sound pressure levels). The segmented regression compared the

summed error of two regressions describing the distribution of the data with a single regression line. Data points for the segmented regression were constrained to consecutively ordered groups of data points. If any combination of consecutively grouped data points resulted in a lower error than the single linear regression, the segmented regression analysis was used to define the I/O function. Linear mixed models were utilized to see if the presence of hearing loss and the frequency tested affected the I/O function slopes or the presence of break-points. For mixed models, we included the subject as a random effect.

Procedures utilized for click-evoked ABR recordings were the same as those discussed for the ASSR above unless otherwise detailed. Six recordings of 1024 epochs were collected in each animal and for each click duration (5, 50, and 100 µs). The 1024 epochs were averaged to produce a grand average ABR waveform, which was subsequently used for ABR peak latency and amplitude measurements. Absolute latencies and amplitudes (P1, N2, P3, P4, and N5) and interpeak latencies (P1-P4, N2-N5, P3-P4) were recorded for each subject at each click duration (5, 50, and 100 µs) by inspection of the ABR waveform. Absolute and interpeak latency values (µs) were plotted as a function of subject age (years) and upper frequency limit of hearing (kHz). Amplitude values (nV) were also plotted as a function of subject age (years) and upper frequency limit of hearing (kHz). Linear mixed models and linear regressions were utilized to determine if any relationships existed between age and the upper frequency limit of hearing (independent variables) and the peak absolute latencies, interpeak latencies, and wave amplitudes.

RESULTS

Animal Description

The subjects included in this study (2 females, 4 males) ranged from 13 – 49 years in age (Table 1). Upper frequency limits of hearing ranged from 48.5 – 137.7 kHz, with 4 animals exhibiting hearing loss when compared to the expected range of hearing in a bottlenose dolphin (bolded, Table 1). Hearing loss was defined in this study as an upper frequency limit of hearing ≤ 100 kHz.

Click Spectra Analysis

The spectra for the 5 μs click ranged from about 20 – 150 kHz (-10 dB point criterion) with peak energy at about 125 kHz and a -10 dB bandwidth of ~57 kHz (~70 – 127 kHz; Figure 3). A lobe energy was also prominent around 55 kHz. A rippling effect was noted as click duration increased from 5 to 100 μs, i.e. the spectra of the click stimulus became less smooth.

Click-Evoked ABR

The click-evoked ABR includes 5 primary components recorded within the first 6 ms post-stimulus onset: P1, N2, P3, P4, and N5 with "P" indicating a positive deflection and "N" indicating a negative deflection. Figure 4 presents ABR recordings produced in response to the 5 μs click (0 dBV, 122 dB SPL) for each animal, ordered by descending upper frequency limit of hearing. Average ABR amplitudes and latencies are listed by animal and waveform component for each click duration in Table 2. Average latency for the first waveform component (P1) was about 1.6 ms and about 3.9 ms for the last waveform component (N5). Average amplitudes ranged from about 163 nV (P1, smallest waveform component) to about 966 nV (N5, largest waveform component). N5 was the dominant wave across all animals whereas P3 was generally the dominant positive wave. Waveform amplitudes generally decreased and latencies increased

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as the upper frequency limit of hearing decreased. However, there were individual differences that cannot be accounted for by the upper frequency limit of hearing alone, e.g. D4 had the lowest amplitude waves and D3 demonstrated shorter latencies than D1.

There was a significant positive relationship between the upper frequency limit of hearing and the amplitude of all waves when considering the ABR to the 5 µs click ($r^2 = 0.73 - 0.81$; $p \leq 0.029$, $\alpha = 0.05$). However, at 50 and 100 µs, this relationship was only maintained for waves P1, N2, and P4 ($r^2 = 0.66 - 0.74$; $p < 0.049$, $\alpha = 0.05$). The relationship was not significant for waves P3 and N5 when produced with the rippled spectra clicks, although the relationship trended in this direction. No relationship between ABR waveform amplitude and age, gender, or animal mass was noted. Similarly, there were no statistically significant relationships between click-evoked ABR latency and age, gender, or upper frequency limit of hearing. Due to a limited sample size, it is probable that the low statistical power of the tests was inadequate to determine if inter-relationships existed between the predictor variables (i.e. there was insufficient sample size for a linear mixed model).

SAM Tone-Evoked ASSR I/O Functions

Slopes of basal I/O functions ranged from 0.5 – 3.5 nV/dB SPL (the stimulus amplitude). The frequency tested significantly affected the slope of the basal I/O function when animal ID was included as a random effect ($p = 0.04$, $\alpha = 0.05$). Whether or not an animal had hearing loss and the upper frequency limit of hearing appeared to have no effect on the basal I/O slope.

Slopes of the second I/O function (following break-point), if present, were always steeper than the basal slope and ranged from 6 – 68 nV/dB SPL. When a break-point was found, it always occurred at stimulus levels > 110 dB SPL. Whether or not a break-point occurred was not predictable based on the presence/absence of hearing loss nor the frequency that was tested.

Figure 5 presents example I/O functions for two subjects at 56 kHz, one demonstrating a break-point (D2, top) and the other without a break-point (D4, bottom).

DISCUSSION

Results Summary

The current study presents subjects with variation in hearing sensitivity (e.g. normal hearing versus hearing loss) and age in an attempt to elucidate any relationship between the click-evoked ABR amplitude and/or latency and the upper frequency limit of hearing. Presbycusis has been demonstrated in the bottlenose dolphin and is observed in subjects D3, D4, and D6 (Houser & Finneran, 2006b). However, despite an increased age, D5 has retained a normal range of hearing and hearing sensitivity. Conversely, despite a younger age, D1 exhibits an unexpected reduction in the range of hearing.

Click-Evoked ABR: Humans versus Bottlenose Dolphins (*Tursiops truncatus*)

In humans, the click-evoked ABR also consists of five primary components labeled waves I, II, III, IV, and V with wave V as the prominent waveform (Figure 6). Wave I is also prominent in Figure 6 due to the recording (electrode) montage (see below for description). Figure 6 represents data collected from a human female participant with normal hearing (thresholds less than 20 dB HL bilaterally, 250 – 8000 Hz). Data collection took place in a double-walled, sound-treated room using a custom-written protocol in LabView. High-intensity clicks at 80 dB SPL re: 20 µPa with a duration of 100 µsec, a rate of 11.1/sec, and alternating polarity were utilized in obtaining 3072 total averages (six recordings of 512 averages). The stimuli were delivered via free-field subwoofer powered by an amplifier (monitored with a small microphone at the entrance of the test ear canal), amplified by 10,000, band-pass filtered (100 –

3000 Hz) and digitized at 25 kHz. The signal rejection level ranged from 15 – 45 µV. The electrode montage was as follows: noninverting (+) at the tympanic membrane (right), common (ground) at the contralateral mastoid (left), and inverting (-) at the forehead. The waveform was inverted and smoothed offline. As Figure 6 demonstrates, the response occurs within the first 10 ms following initial stimulation, and neuroanatomical correlates are as follows: distal portion of cranial nerve eight (wave I) -> proximal portion of cranial nerve eight (wave II) -> cochlear nucleus and superior olivary complex (wave III) -> multiple generator sites including the lateral lemniscus and inferior colliculus (waves IV and V) (Jewett & Williston, 1971). Qualitatively, a decrease in amplitude and an increase in latency occur with decreasing stimulus intensity (i.e. nearing threshold) or increasing stimulus rate (e.g. 11.1/sec to 27.1/sec). The click-evoked ABR in the bottlenose dolphin is similar to other terrestrial mammals (including humans) in response generation site, onset, and duration. However, it occurs slightly earlier (6 ms as opposed to 10 ms in humans) and is larger in amplitude, likely due to an increased concentration of OHCs and IHCs in combination with more densely innervated IHCs (Ketten, 1997).

Clinically, the click-evoked ABR can be used in neurodiagnostic assessment of suspected retrocochlear pathology (i.e. cranial nerve VIII or brainstem lesions), hearing sensitivity estimation for noncompliant children and adults, neonatal hearing screenings, intraoperative monitoring of cranial nerve VIII and auditory brainstem function, and diagnosis of auditory neuropathy spectrum disorder.

Numerous subject factors influence click-evoked ABR variability in humans, including age (i.e. neural development), gender, body temperature, ototoxic medication(s), noise exposure, and hearing sensitivity. The ABR does not mature until around 18 months of age due to incomplete nerve fiber myelinization, reduced axon diameter, and immature synaptic functioning

(Hall, 2007). Increased amplitudes and decreased latencies are recorded in females versus males at all ages. Although the etiology of this gender difference remains unknown, speculations regarding better average hearing sensitivity and higher average body temperature in females versus age-matched males are most commonly encountered in the literature (Hall, 2007). An additional explanation involves the average smaller head size and brain dimensions in females versus males (Hall, 2007). This relates to the click-evoked ABR in that latencies will be shorter with decreased distance between neural generators and amplitudes will be increased with shortened distance from the recording electrode to the neural generator (Hall, 2007). Hormonal fluctuations throughout the female menstrual cycle are also thought to play a role in the trends described above with latency of wave V correlated with levels of ovarian steroids (Elkind-Hirsch, Wallace, Malinak, & Jerger, 1994; Caruso et al., 2003). Decreased amplitudes and increased latencies are also noted with extreme changes in body temperature (i.e. hyperthermia and hypothermia) (Legatt, 2002). Lowered temperature results in delayed synaptic transmission and a decrease in axonal conduction velocity (Benita & Conde, 1972; deJesus, Hausmanowa-Petrusewicz, & Barch, 1973). Extreme hypothermia (body temperature $< 14 - 20°C$) causes the ABR to disappear (Rosenblum, Ruth, & Gal, 1985). Increased temperature and the ABR has been less well studied and experimental manipulations are often limited to a few degrees Celsius (Hall, 2007). A small group of studies has shown decreased amplitude and latency values with increasing body temperature (Barnett, 1980; Gold, Cahani, Sohmer, Horowitz, & Shahar, 1985). Exposure to ototoxic medications and noise most often damages the cochlear OHCs with the basal region being most susceptible, resulting in a high frequency, (typically) sensory hearing loss.

With increasing severity of hearing loss (i.e. larger magnitude of loss affecting wider range of high frequencies), amplitudes decrease and latencies increase. Clinical, diagnostic patterns in amplitude and latency values are observed when using the click-evoked ABR in identification of hearing loss type. For conductive losses (CHL, i.e. external and/or middle ear pathologies), absolute latencies are delayed due to increased conduction time from the middle ear (tympanic membrane and ossicles) to the inner ear whereas inter-peak latencies remain within normal limits (suprathreshold and threshold levels). For sensory losses (HC and/or synaptic lesions), absolute latencies are normal and inter-peak latencies remain either within normal limits or slightly shorter at suprathreshold levels. However, at threshold levels, absolute latencies are delayed for all waveform components, particularly waves I and V. Given that most sensory hearing losses affect the high frequencies, delayed absolute latencies are expected because the (high frequency) click-evoked response is now generated from a more apical (low frequency) region of the cochlea and requires longer "travel time" along the basilar membrane before sensory transduction at the HCs (Hall, 2007). For neural losses (spiral ganglion fibers and beyond), both absolute and inter-peak latencies are delayed at suprathreshold levels due to a lack of neural synchrony. Waveform morphology is typically grossly abnormal for the same reason. Many of the subject factors common to humans presumably also affect click-evoked ABR variability in the bottlenose dolphin. High frequency hearing loss with age, defined as presbycusis, has been documented in the bottlenose dolphin (Houser & Finneran, 2006b) and likely contributes to decreased ABR amplitudes and increased wave latencies (exhibited by subjects D3, D4, and D6 in this study). Furthermore, Houser and Finneran (2006b) documented presbycusis occurring earlier in males versus females. Similar to terrestrial mammals, audition appears to be a genetically regulated process in the bottlenose dolphin (i.e. passed from parent to

offspring) (Houser & Finneran, 2006b). This trend could explain the early-onset hearing loss exhibited by subject D1 in the present study given that his father also demonstrated early-onset hearing loss. Genetic etiology is further supported by the fact that D1 has had no major illnesses throughout his lifetime and has never been prescribed an ototoxic medication, known to result in high frequency hearing loss in at least one odontocete (*Delphinapterus leucas*) for which aggressive treatment was required (Finneran et al., 2005). Noise exposure is also a subject factor known to cause high frequency (typically) sensory hearing loss, but due to the fact that all subjects included in this study live in the same area, it is assumed their noise exposure history is similar.

Loudness Growth I/O Function Trends based upon Hearing Sensitivity

Loudness can be defined as the subjective perception of sound pressure amplitude. In humans, loudness can be categorized behaviorally to generate tonal functions allowing analysis of loudness growth with increasing intensity (Brand & Hohmann, 2001). Furthermore, these I/O functions differ notably between individuals with normal hearing, CHL, and sensorineural hearing loss (SNHL) (Figure 7). For those with typical hearing, loudness growth is roughly linear on a logarithmic scale at low and moderate intensities with compressive loudness growth at high intensities due to neuronal saturation (Marozeau & Florentine, 2007). Those with CHL exhibit attenuated functions that remain parallel to loudness growth curves for those with typical hearing (i.e. roughly linear on a logarithmic scale with compressive growth at high intensities) (Marozeau & Florentine, 2007). Individuals with SNHL generate functions that steeply slope from threshold to mid-level intensities due to higher intensity needed for neuronal stimulation. At high intensities, these functions resemble those of typical hearing individuals (i.e. compressive growth) (Marozeau & Florentine, 2007).

Loudness growth can also be predicted using electrophysiologic measures such as the ASSR. Emara and Kolkaila (2010) examined ASSR amplitude as a correlate of loudness at 500, 1000, 2000, and 4000 Hz in study participants with normal hearing and moderate, bilateral SNHL. For participants with normal hearing, ASSR amplitude increased more rapidly above 70 dB SPL re: 20 µPa at all test frequencies. Participants with moderate, bilateral SNHL showed a more rapid increase in ASSR amplitude above 60 dB SPL re: 20 µPa. The authors suggested the rapid increase in ASSR amplitude at higher intensities could be due to greater spread of basilar membrane activation, creating additional IHC activation in combination with OHC activation present at low intensities (Emara & Kolkaila, 2010). Emara and Kolkaila (2010) also found a significant correlation between behavioral loudness rankings and electrophysiological ASSR amplitudes at 1000, 2000, and 4000 Hz.

In the current study, loudness growth input/output functions also using ASSR amplitude as a correlate of loudness demonstrate what is thought to be abnormal loudness recruitment at higher sound pressure levels (i.e. slope increases more quickly at levels > 110 dB SPL as opposed to lower sound pressure levels) in bottlenose dolphins with normal or reduced high frequency limit of hearing.

Tursiops truncatus: Potential Applications of the Click-Evoked ABR in Combination with the SAM Tone-Evoked ASSR

Given the statistically significant relationship between click-evoked ABR waveform amplitudes (P1, N2, P3, P4, and N5) and upper frequency limit of hearing at the shortest click duration (5 µs), this electrophysiological method holds potential clinical application, particularly in a screening context. With further study defining normative values for response amplitude based upon upper frequency limit of hearing, a screening protocol utilizing the click-evoked

ABR could be implemented for routine hearing assessment monitoring in those animals under human care (i.e. comparing to baseline testing). An additional and significant application could be auditory monitoring for those animals receiving ototoxic antibiotics such as gentamycin or amikacin, loop diuretics such as furosemide, and/or platinum-based chemotherapy agents such as cisplatin known to result in SNHL. However, it should be noted that the click-evoked ABR methodology lacks frequency specificity in assessment; therefore, the SAM tone-evoked ASSR methodology should be used for initial assessment, following any significant changes in the click-evoked ABR response (i.e. outside of test/re-test reliability), and/or if there is trainer concern regarding the animal's response to auditory cues or detriment in localization ability.

For animals in the wild, utilizing the click-evoked ABR methodology as a screening tool would be especially applicable given the ability to examine a wide range of frequencies with one stimulus and its short test time (about 30 seconds as opposed to about 30 minutes using the SAM tone-evoked ASSR methodology).

CONCLUSIONS

Visual inspection of the click-evoked ABR suggests that relationships between the waveforms and hearing capabilities exist. However, with a limited sample size, even though it appears that trends may exist with respect to hearing capabilities and I/O functions, there is enough variability within and among subjects to limit our ability to statistically measure the relationships. Stimuli such as clicks with adjusted, equivalent acoustic energy across the bottlenose dolphin's auditory filters could be used to refine the current methodology and provide a better indication of frequency-specific cochlear pathology. Nevertheless, further study is

needed to establish normative data by species for widespread use in the marine mammal

veterinary clinic, research complex, and/or field.

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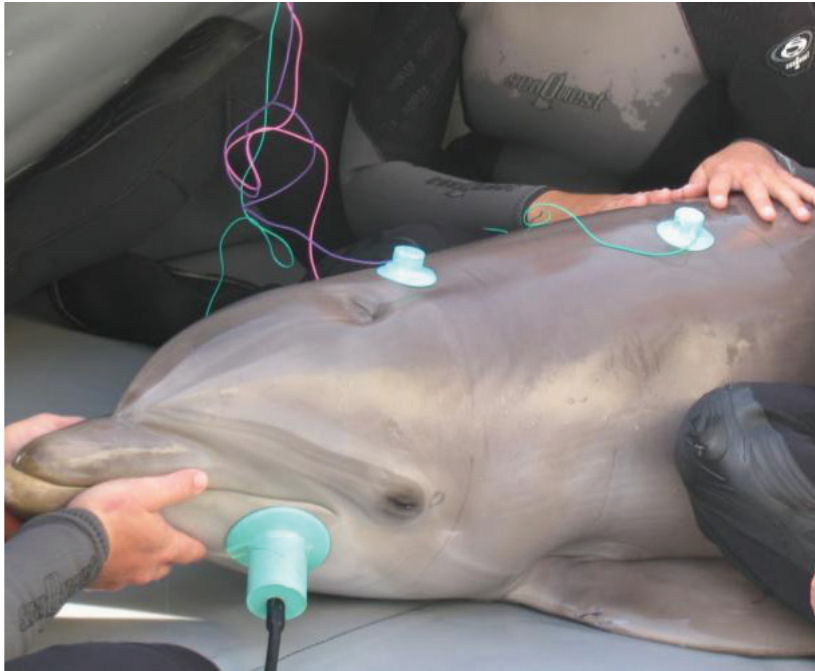
FIGURES AND TABLES

Figure 1: Auditory-evoked potential (AEP) electrode montage and jawphone placement

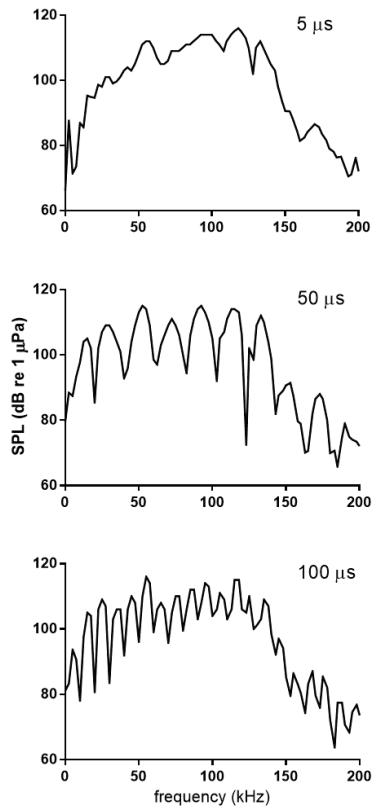Figure 2: Example AEP data collection session

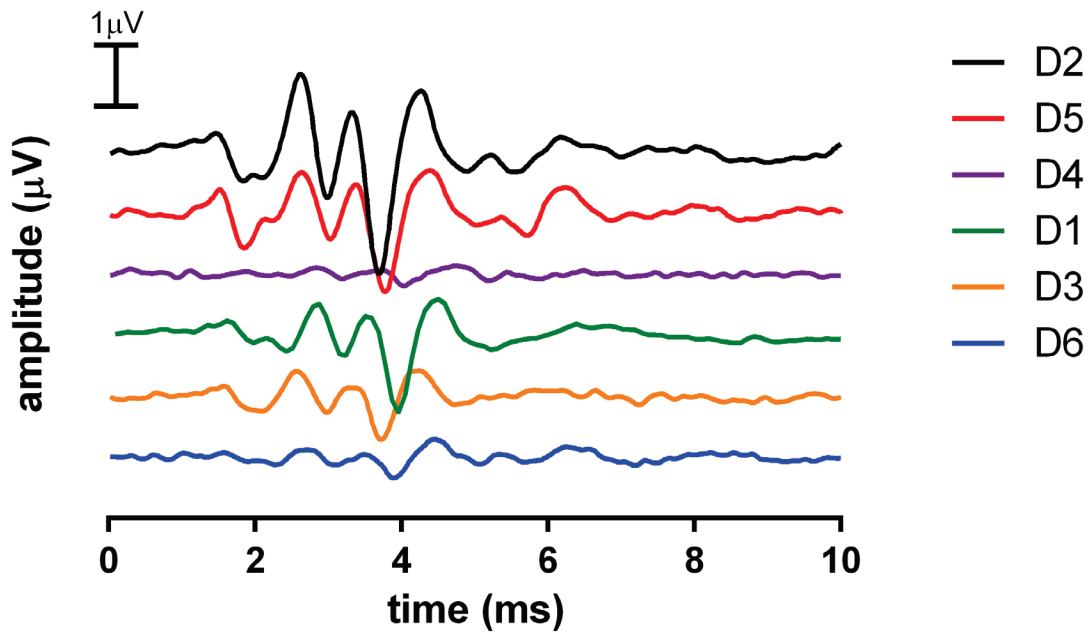Figure 3: Click spectra by duration (5, 50, and 100 μs)

Figure 4: Dolphin click-evoked auditory brainstem responses (ABR) (0 dBV, 122 dB SPL re: 1 μPa) ordered by descending upper frequency limit of hearing
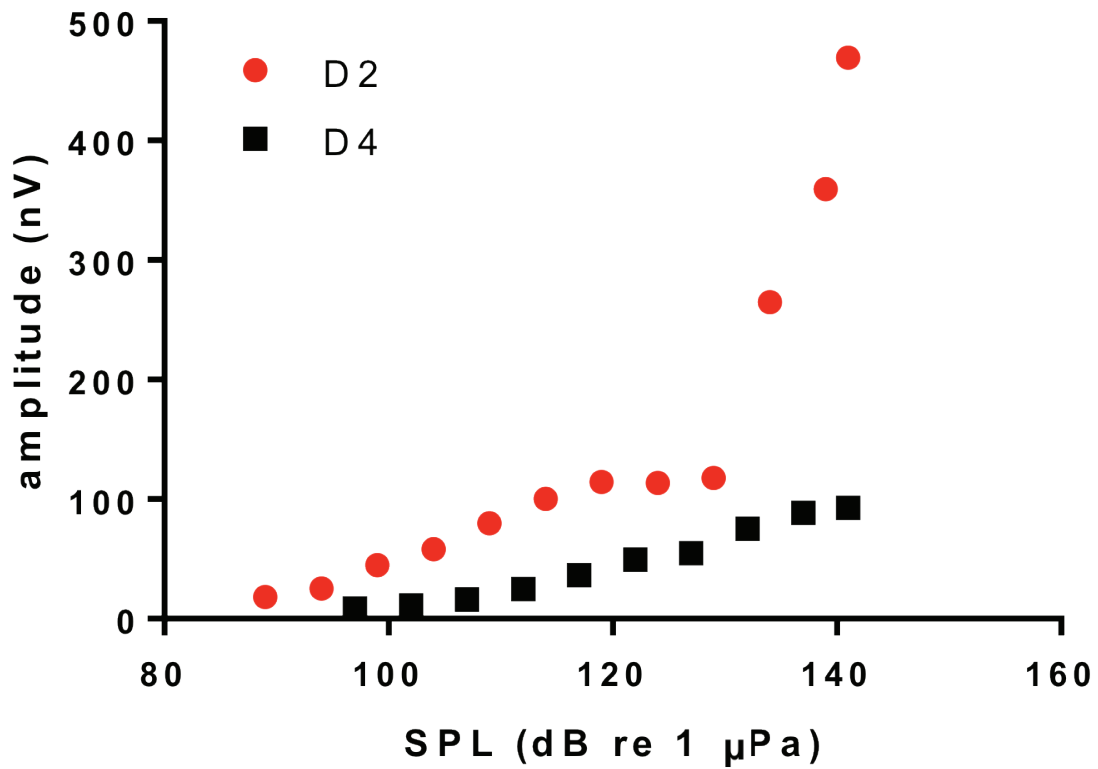
Figure 5: Sinusoidal amplitude-modulated (SAM) tone-evoked auditory steady-state response (ASSR) input/output (I/O) functions at 56 kHz, D2 (top, break-point) and D4 (bottom, no break-point)
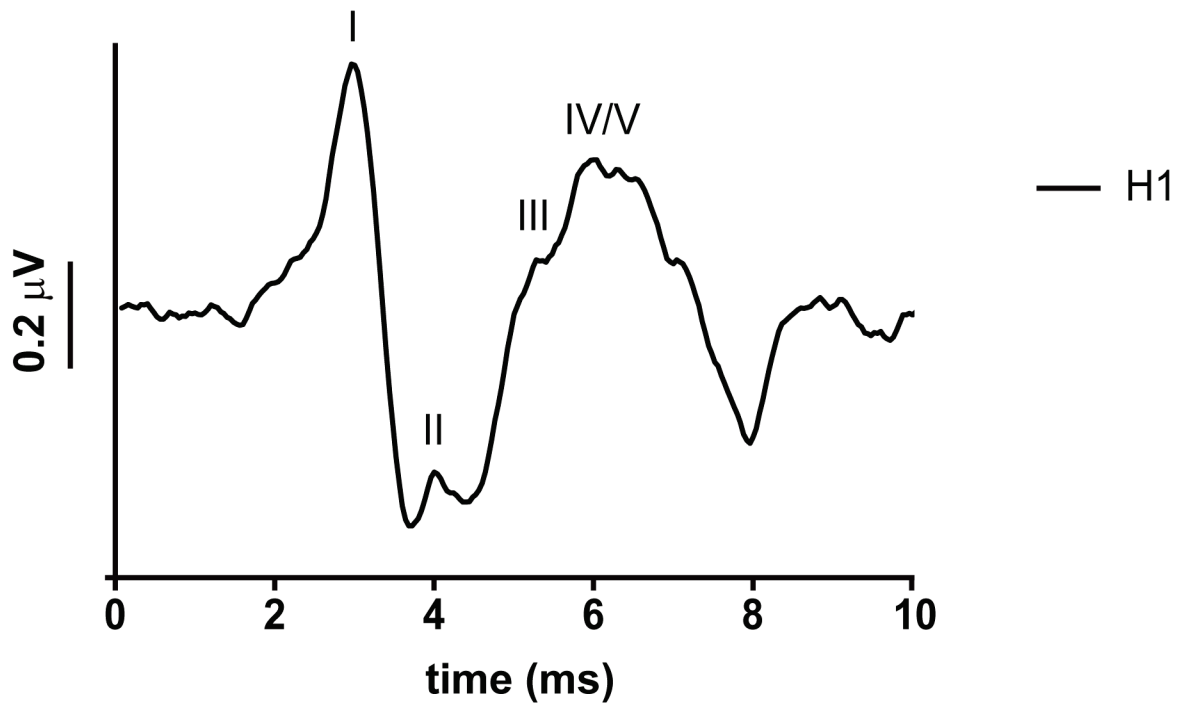
Figure 6: Human click-evoked ABR (80 dB SPL re: 20 μPa)

Categorical Loudness Scaling (4 kHz)
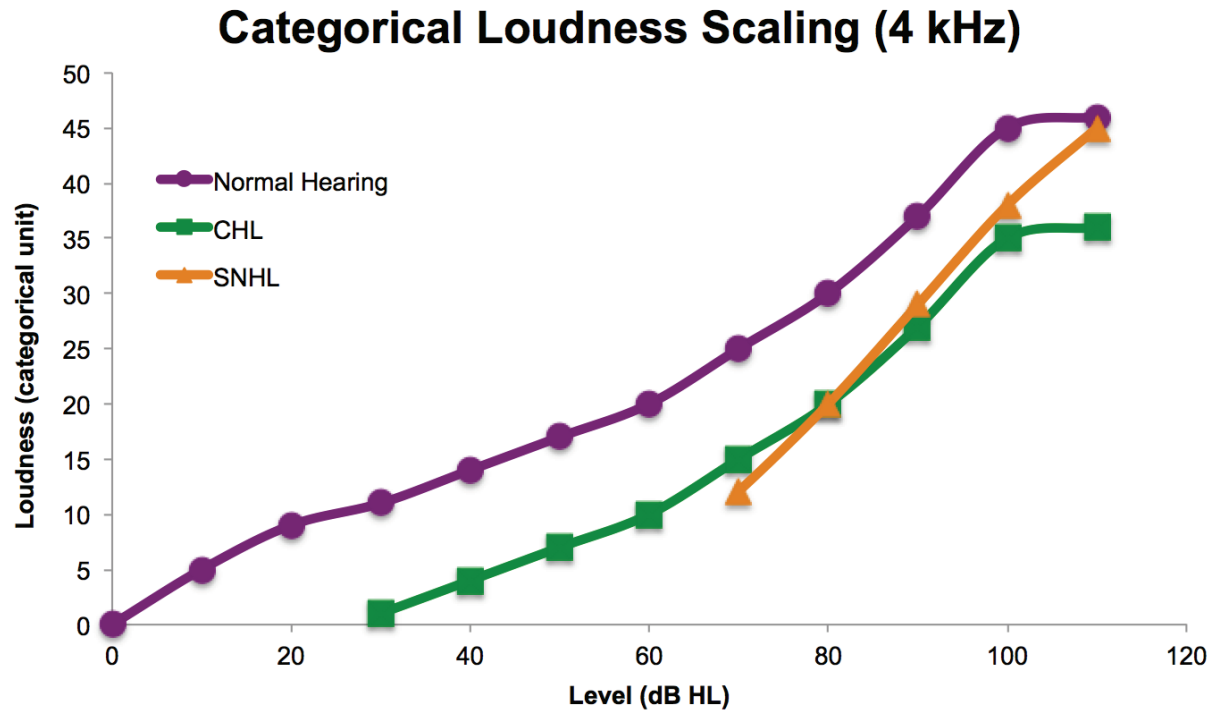
Figure 7: Example categorical loudness judgments at 4 kHz representing individuals with normal hearing, conductive hearing loss (CHL), and sensorineural hearing loss (SNHL) adapted from data presented in Brand and Hohmann (2001)

Table 1. Subject Demographics				
Animal ID	Gender	Age (Years)	Weight (lbs)	Upper Frequency Limit of Hearing (kHz)
D1	Male	13	435	**68.4**
D2	Male	22	400	137.7
D3	Male	30	419	**50.3**
D4	Male	33	414	**82.8**
D5	Female	35	538	128.1
D6	Female	49	462	**48.5**

Table 2. Click-Evoked ABR: Average Amplitudes and Latencies									
5 µs									
Amplitude (nV)					Latency (ms)				
Animal	P1	N2	P3	P4	N5	P1	N2	P3	P4	P5
D1	55.21	163.7	340.6	268.1	839.0	1.63	2.41	2.82	3.54	3.97
D2	344.3	535.8	1334.7	747.2	2182.2	1.45	1.79	2.59	3.30	3.67
D3	127.3	187.3	362.1	185.9	643.9	1.56	2.05	2.53	3.30	3.70
D4	32.20	71.89	87.98	78.28	171.6	1.75	2.20	2.89	3.59	4.07
D5	394.2	769.9	785.5	555.8	1596.5	1.49	1.83	2.64	3.34	3.75
D6	26.37	55.18	80.09	74.73	130.8	1.78	1.98	2.60	3.58	3.97
Average	**163.3**	**297.3**	**498.5**	**318.3**	**927.3**	**1.61**	**2.04**	**2.68**	**3.44**	**3.85**
SD	**150.0**	**264.5**	**441.3**	**250.8**	**743.4**	**0.12**	**0.21**	**0.13**	**0.13**	**0.15**

50 µs									
Amplitude (nV)					Latency (ms)				
Animal	P1	N2	P3	P4	N5	P1	N2	P3	P4	P5
D1	125.0	174.9	381.6	286.9	948.4	1.63	2.42	2.85	3.54	3.97
D2	317.1	462.5	1207.7	648.5	2009.7	1.47	1.85	2.63	3.33	3.70
D3	171.1	233.5	482.2	192.1	813.9	1.55	2.13	2.58	3.42	3.71
D4	39.73	61.66	84.18	63.62	202.0	1.80	2.21	2.91	3.63	4.07
D5	342.2	632.9	667.0	452.2	1297.7	1.50	1.85	2.65	3.38	3.77
D6	64.28	101.3	141.9	119.1	325.9	1.70	2.15	2.75	3.51	3.92
Average	**176.6**	**277.8**	**494.1**	**293.7**	**932.9**	**1.61**	**2.10**	**2.73**	**3.47**	**3.86**
SD	**127.4**	**223.9**	**410.8**	**221.3**	**664.8**	**0.13**	**0.22**	**0.13**	**0.11**	**0.15**

100 µs									
Amplitude (nV)					Latency (ms)				
Animal	P1	N2	P3	P4	N5	P1	N2	P3	P4	P5
D1	125.0	213.7	475.3	333.7	1051.0	1.61	2.43	2.87	3.56	3.97
D2	358.6	542.6	1360.5	685.6	2208.2	1.47	1.86	2.63	3.34	3.69
D3	178.1	220.0	455.3	190.9	705.4	1.50	2.12	2.60	3.35	3.74
D4	53.68	65.85	81.69	73.44	200.8	1.83	2.36	2.87	3.69	4.08
D5	341.8	639.9	623.2	473.8	1251.2	1.51	1.87	2.68	3.37	3.78
D6	76.02	106.2	154.6	117.0	377.4	1.75	2.10	2.78	3.58	3.96
Average	**188.9**	**298.0**	**525.1**	**312.4**	**965.7**	**1.61**	**2.12**	**2.74**	**3.48**	**3.87**
SD	**132.2**	**236.9**	**458.0**	**234.9**	**725.5**	**0.15**	**0.24**	**0.12**	**0.15**	**0.16**