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When signal meets noise: Immunity of the frog ear to interference

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Abstract

Sound stimulates the tympanic membrane (TM) of anuran amphibians through multiple, poorly understood pathways. It is conceivable that interactions between the internal and external inputs to the TM contribute to the non-linear effects that noise is known to produce at higher levels of the auditory pathway. To explore this issue we conducted measurements of TM vibration in response to tones in the presence of noise in the frog *Eupsophus calcaratus*. Laser vibrometry revealed that the power spectra ($n=16$) of the TM velocity in response to pure tones at a constant level of 80 dB SPL had a maximum centered at an average frequency of 2344 Hz (range 1700–2990 Hz) and a maximum velocity of 61.1 dB re 1 $\mu\text{m/s}$ (range 42.9–66.6 dB re 1 $\mu\text{m/s}$). These TM vibration velocity response profiles in the presence of increasing levels of 4-kHz band-pass noise were unaltered up to noise levels of 90 dB SPL. For the relatively low spectral densities of the noise used, the TM remains in its linear range. Such vibration patterns facilitate the detection of tonal signals in noise at the tympanic membrane, and may underlie the remarkable vocal responsiveness maintained by males of *E. calcaratus* under noise interference.

Keywords

Anurans; Noise exposure; Tympanic membrane

Introduction

The inner ear of anuran amphibians is stimulated through multiple pathways. Sound impinging directly on the tympanic membrane (TM) is effective in exciting the receptors of the inner ear. However, the amphibian ear acts as a sound-pressure-gradient receiver in which the incoming signal impinges on both the external and internal surfaces of the TM and it is the pressure difference across the eardrum that is its driving force (Chung *et al.* 1978; Pinder and Palmer 1983; Palmer and Pinder 1984). In addition, accessory pathways also play an important role in sound reception. A pathway involving the body wall overlying the lungs was first reported in the neotropical frog *Eleutherodactylus coqui* (Narins *et al.* 1988). The route of sound transmission proposed by these authors involves the lungs, glottis, mouth cavity and the internal surface of the TM. This pathway has subsequently been shown to exist in several other tree frogs and dendrobatids (Ehret *et al.* 1994). In addition, airborne sound is transmitted to the inner ear by extratympanic pathways, which are particularly important at frequencies below

around 1 kHz (Lombard and Straughan 1974; Wilczynski *et al.* 1987). Such routes are fundamental for sound reception in species lacking a TM (Lindquist *et al.* 1998; Hetherington and Lindquist 1999). However, in spite of studies characterizing these different pathways for sound transmission to the inner ear, the interaction between the tympanic and extratympanic inputs to the inner ear remains poorly understood and it is not clear whether they combine their inputs in linear or nonlinear modes (Lewis and Narins 1999).

Noise is a ubiquitous source of interference for animals that communicate acoustically. To exchange signals in noisy conditions, various vertebrates, including humans, have developed adaptive behaviors such as increasing the intensity, periodicity or duration of their vocalizations (Narins and Zelick 1988; Cynx *et al.* 1998; Brumm and Todt 2002; Pytte *et al.* 2003; Brumm 2004; Brumm *et al.* 2004; Brumm and Slabbekoorn 2005; Penna *et al.* 2005; Sun and Narins 2005; Penna and Hamilton-West 2007). Other animals shift the frequency components (and their spectral sensitivities) up above the offending noise (Slabbekoorn and Peet 2003; Narins *et al.* 2004) to maintain a high signal-to-noise ratio and thus ensure effective communication (Feng *et al.* 2006).

Noise can have contrasting effects on the auditory system: auditory nerve fibers typically increase their response thresholds to pure tones, shifting their rate-level functions to higher sound pressure levels and compressing their dynamic ranges in response to tones (Ehret and Capranica 1980; Hillery and Narins 1984; Narins 1987; Narins and Wagner 1989; Lin and Feng, 2001). Midbrain auditory neurons in the torus semicircularis also typically show increases in thresholds of response to pure tones and a rightward shift of their rate level functions in the presence of noise (Ratnam and Feng 1998). Such changes are large relative to those produced in auditory nerve fibers (Lin and Feng, 2001). However, under certain circumstances, auditory function is enhanced at the periphery in the presence of low or moderate noise levels (Henry 1999; Indresano *et al.* 2003; Nadrowski *et al.* 2004). In frogs, midbrain auditory neurons respond with higher discharge rates (Ratnam and Feng 1998), or greater synchrony (Bibikov 2002) to stimuli accompanied by broadband noise at low levels. These results have been attributed to stochastic resonance, a nonlinear effect that improves the detection and transmission of weak coherent signals at the cellular membrane level (e. g.: Douglas *et al.* 1993; Benedix *et al.* 1994; Wiesenfeld and Moss 1995). These different effects of noise on the auditory function represent nonlinearities that shape the processing of acoustic signals at different levels of the auditory pathway (Robles and Ruggero 2001).

Males of *Eupsophus calcaratus*, a leptodactylid frog from the temperate forests of southern Chile exhibit an interesting behavior in the presence of noise. These frogs increase their call rate remarkably and maintain their responsiveness to synthetic calls when exposed to pre-recorded natural noises at moderate intensities. They also behave similarly in response to a band-pass noise encompassing the main spectral components of the conspecific advertisement call at moderate and high intensities (66 to 78 dB SPL; Penna *et al.* 2005). Such a phenomenon implies the preservation of the capability of detection of acoustic signals amid noise which could be fostered by nonlinearities along the auditory pathway facilitating responses to signals in noise (e.g. Ratnam and Feng 1998; Bibikov 2002). Alternatively, such a behavioral response could result from linear modes of response adequate for preserving signal detection.

To date, there are no reported data from frogs on the effect of noise on the response of the TM to pure tones. Because the multiple pathways of sound to the tympanum are often ill-defined and not entirely understood, it is not clear if interactions at this level contribute to the non-linear effects that noise produces at higher levels of the auditory pathway. The present study explores the vibratory response of the TM of *E. calcaratus* to pure tones in the presence of various levels of broad-band noise. We intend to determine the mode of response of the TM as it may relate to the effects of noise on the vocal behavior of this species.

Materials and methods

Animal preparation

Eleven healthy adult males (average snout-vent length: 39.5 mm; range: 37–42 mm) and five females (average snout-vent length: 41.8 mm; range: 39 – 44 mm) of *Eupsophus calcaratus* were captured in their natural habitat in the Vicente Pérez Rosales National Park (Chile) in October 2003 and 2004. The animals were housed in terraria with plants, fresh soil and water in a refrigerated room at 8–10°C, within the temperature range found in these frogs' natural habitat. The frogs were weighed (Acculab 333, Acculab, Göttingen, Germany) to the nearest 0.1 g and their snout-vent length measured to the nearest mm before being deeply anesthetized by immersion in a 0.4 % tricaine methanesulfonate salt (MS-222) solution. For the levels of anesthesia used, the frogs typically showed occasional movement of the vocal sac. The anesthetized animals were placed on a Peltier plate in a natural position (dorsal side up) on a custom-made vibration-damped table in a sound-attenuating chamber. A DC current of 1–1.2 A was supplied to the Peltier device in order to keep the frog's body at 8–10°C. Cloacal temperatures were monitored with a miniature thermometer probe (Duolog Digisense, Digisense Inc., Redwood City, California, USA). The frogs were moistened frequently to prevent desiccation and to facilitate cutaneous respiration. The experiments were conducted in the Laboratory of Neuroethology, University of Chile (Protocol CBA# 061 FMUCH).

Laser measurements

The intensity of the delivered sounds was monitored with a 1/2" microphone (Brüel and Kjær 4155, Brüel and Kjær, Nærum, Denmark) and preamplifier (Brüel and Kjær ZC 0020) positioned above the head of the frog at approximately 3 mm from the dorsal margin of the left TM. The preamplifier was connected with an extension cable to a sound-level-meter (Brüel and Kjær 2230) placed outside the sound-attenuating chamber.

Pure tones of 750-ms duration and 100-ms rise-fall times were synthesized with SigGen software (Tucker-Davis Technologies, TDT, Alachua, Florida, USA) running on a Pentium II computer. The frequencies were increased from 0.1 to 1.0 kHz in 100-Hz steps, from 1.0 to 4.0 kHz in 50-Hz steps, and 4.1 kHz. The output of the computer's 16-bit D/A board (DA, TDT) was controlled with a programmable attenuator (PA4, TDT). Continuous white noise was produced with a waveform generator (WG1, TDT) and band-pass filtered (0.1 kHz and 4.1 kHz cutoff frequencies, PF1, TDT). The filter output was passed through a manual attenuator (Hewlett-Packard 350D, Hewlett-Packard Company, Palo Alto, California, USA) and a custom-designed passive adder in which the noise was mixed with the pure-tone signal. The tone-plus-noise signal was amplified (NAD C 320 BEE, NAD Electronics, Pickering, Ontario, Canada). The signal was delivered using a two-way loudspeaker (Dynaudio BM 5, Dynaudio, Skanderborg, Denmark) with a flat response (± 3 dB) between 43 Hz and 20 kHz, which was placed at 100 cm in front of the experimental subject. All measurements were made on the left TM. A calibration was performed by measuring the sound pressure levels (SPLs) for all tones at a fixed amplification, at the beginning of each experimental session. These measurements were constant within ± 13 dB and provided reference values for the programmable attenuator at the different frequencies tested, in order to ensure that all tones were delivered at a constant intensity of 80 ± 1 dB RMS fast SPL. Pure tones were presented at a rate of one stimulus per second in order of increasing frequency. The tone sequence was presented first in the absence of generated noise, and then accompanied by increasing noise levels (50 to 80 dB RMS SPL, for all sixteen subjects and 50 to 90 dB RMS SPL for eight subjects, 10-dB steps). For three individuals, pure tones of a frequency near the "best" TM frequency were presented at increasing intensities (50 to 104 dB SPL RMS, 6-dB steps). The best TM frequency was defined as the frequency of maximum TM vibration for a constant amplitude (80 dB SPL) pure-tone stimulus (1.5 – 2.5 kHz, depending on the subject).

A single-point laser vibrometer sensor head (Polytec OFV-303, Polytec, Waldbronn, Germany) was positioned at approximately 60 cm from the animal's head. The laser beam was aimed at the center of the TM perpendicular to its surface, using a binocular light microscope (PZO OP-1, PZO, Warsaw, Poland) fitted with a 400-mm objective lens. The beam placement for conducting measurements was set after scanning the TM surface while delivering a tone at a frequency producing the highest vibrometer output at approximately the center of the TM. Subsequent placements confirmed the stability of the response. The laser-sensor head was connected to a vibrometer controller processor (Polytec OFV-3001), which produced a peak voltage output proportional to the vibration velocity of the structure being measured. As a control for each TM measurement, the velocity of the bony surface immediately dorsal to the tympanic surface was recorded.

Data acquisition and analysis

The output from the vibrometer controller was fed to the left channel of an analog tape recorder (Uher, 4400 Report Monitor, Uher Werke, München, Germany). The right channel was used to record comments and experimental settings. The equipment for data recording and analysis was calibrated so that the vibration measured by the laser and stored on tape could be converted to absolute velocity values. This was done in each experimental session by delivering a tone of approximately the best TM frequency (the best TM frequency was determined after off-line analysis of the data) and measuring the peak amplitude of the vibrometer output (mV) directly on an oscilloscope. This signal was subsequently recorded with the same recording level of the tape recorder used for the responses to the entire sequence of tones from which the TM-velocity profile was obtained. No recordings of the vibrometer output were made for the presentations of noise at 90 dB SPL because in the presence of this high noise level, the tones were recorded directly to the tape recorder in order to be able to track the intervals during which the tones were presented.

The stored laser analog signal was digitized [Power PC G4 Macintosh computer (Apple Computers Inc., Cupertino, California, USA) using Peak 2.52 software (Berkeley Integrated Audio Software, Petaluma, California, USA)] at a 44-kHz sampling rate, using an anti-aliasing filter (FT6-2, TDT) and an analog-digital interface (Motu 828, Motu, Cambridge, Massachusetts, USA). Power spectra of the digitized signals were obtained with the Signalyze 3.12 software (Network Technology Corporation, Charlestown, Massachusetts, USA). The best TM frequency was estimated by sweeping the stimulus from low to high frequencies and monitoring the waveform at the laser output for maximum amplitude. Next, the relative amplitude (in dB) of the dominant spectral peak for the best TM frequency was determined by performing a FFT on the waveform at the laser output. This amplitude was then used as a reference for all subsequent spectral peaks measured over the range tested, to obtain plots of the peak TM velocity as a function of frequency (TM-velocity profiles).

To assess the variation of the TM response to pure tones in the presence of noise, correlation coefficients were calculated between velocity values measured for the different tone frequencies in the absence of generated noise and during exposures to band-pass noise at five levels. The correlation coefficients were calculated from the velocity values (dB re 1 $\mu\text{m/s}$) aligned for the different tone frequencies under the six different treatments. In addition, a center frequency of TM vibration was calculated from velocity values within 20 dB of the maximum velocity measured for a TM velocity profile in response to 80 dB SPL pure tones in the absence of generated noise and in the presence of band-pass noise at different levels. The center frequency was obtained by calculating the area under the curve [sum of velocities (dB) * frequency (Hz)] divided by the sum of the velocities (dB) at the frequencies considered. This algorithm has been used to calculate center frequencies of audiograms based on multi-unit neuronal recordings in the anuran torus semicircularis (Penna et al. 2008).

Results

The power spectra ($n=16$) of the TM velocity in response to pure tones of frequencies of 0.1–4.1 kHz at a constant level of 80 dB SPL, showed a maximum centered at an average frequency of 2344 Hz (range: 1700–2990 Hz). The maximum velocity averaged 61.1 dB re 1 $\mu\text{m/s}$ (range: 42.9–66.6 dB re 1 $\mu\text{m/s}$). These values were not correlated with animal size or weight (Pearson correlation, $p > 0.126$ for all correlations). Figure 1 shows waveforms and sound spectra in different noise levels with the microphone at the position of the left tympanum of one individual. Figure 2 shows the waveforms and spectra obtained from the vibrometer output.

The velocity profiles of the TM vibration velocity in response to 80 dB RMS SPL pure tones in the absence of generated noise (Fig. 3a) and in the presence of band-pass noise at different levels (Fig. 3b–f) were rather similar for the range of frequencies tested. A dip in the TM vibration velocity was typically observed at approximately 1400 Hz. The vibration velocity of the bony eminence dorsal to the TM in response to 80 dB RMS SPL pure tones typically was approximately 30 dB below the maximum TM velocity (Fig. 3g). The TM vibration velocity in response to pure tones of approximately the best frequency of the TM presented at increasing intensities was linear over at least a 50 dB range, as shown in Fig. 4.

The variation of the TM vibration velocity profiles was assessed with the Pearson correlation coefficient of the TM vibration velocity in response to pure tones between measurements in the absence of generated noise and in the presence of five different levels of band-pass noise. All correlation coefficients calculated ($n=72$) were significant ($p < 0.001$) and showed no significant differences considering either the 16 subjects tested at noise intensities from 50 to 80 dB SPL (ANOVA for repeated measures, $F_{3,45} = 0.290$, $p = 0.833$) or the 8 subjects tested at noise intensities from 50 to 90 dB SPL ($F_{4,28} = 1.042$, $p = 0.401$) (Fig. 5a). The variation in the TM-vibration-velocity profiles was also measured with the center frequency calculated from the area under the curve for velocity values within 20 dB of the maximum velocity. This measure did not differ significantly between measurements in the absence of generated noise and exposures to different bandpass noise levels, considering either the 16 subjects tested at noise intensities from 50 to 80 dB SPL (ANOVA for repeated measures, $F_{4,60} = 1.334$, $p = 0.268$) or the 8 subjects tested at noise intensities from 50 to 90 dB SPL ($F_{5,35} = 1.94$, $p = 0.112$) (Fig. 5b).

Discussion

Tympanum vibration measurements of *E. calcaratus* reveal that the response of the TM center to pure tones is unaltered over a wide range of behaviorally relevant broadband-noise levels. Maximum vibration amplitude of the TM center was obtained for a tone of approximately 2400 Hz, which corresponds closely to a prominent spectral peak of the advertisement call of this species (Penna 2004; Márquez *et al.* 2005). This correspondence suggests that sound input via the tympanic membrane is important for detection of signals of biological significance in this species. The average maximum TM velocity of approximately 60 dB re 1 $\mu\text{m/s}$ obtained in response to tones of 80 dB SPL is similar to values shown in a previous study of *Rana catesbeiana* (0 dB re 1 mm/s = 60 dB re 1 $\mu\text{m/s}$) in response to tones of 90 dB SPL (Mason and Narins 2002; Figs. 3, 7 and 8). This comparison indicates that the transfer function of the TM of *E. calcaratus* has a gain 10 dB higher than *R. catesbeiana*. The dip in TM velocity at approximately 1400 Hz resembles those obtained in previous studies with different anuran species measured with their glottis closed (Jørgensen *et al.* 1991) and lungs inflated (Jørgensen 1991), the same state as the animals used in the current study.

The results showing an invariance of the velocity vibration profiles in response to pure tones indicate that the TM center point exhibits a linear mode of response. The spectral density (dB/

Hz) of the band-pass noise with a 4 kHz bandwidth is 36 dB below the tone SPL (which corresponds to its spectral density). Therefore, at the maximum noise level used (90 dB SPL) the energy in the masking noise at the frequency of the tone was still 26 dB (i.e. 20 times) below the tone level of 80 dB SPL (= 80 dB/Hz). In a linear system no significant changes would be expected to result with the addition of such low spectral density levels. Our results are in agreement with indirect evidence for a linear mode of response of the anuran tympanic membrane as reported by Anson *et al.* (1985), and direct evidence from laser measurements demonstrating a linear response over a 20 dB stimulus dynamic range (Ho and Narins 2006). It is particularly interesting that such a linear response is maintained, since it has been shown that the TM vibration is activated by selective stimulation of spots in the lateral body wall (Jørgensen *et al.* 1991; Ehret *et al.* 1994). These spots themselves are tuned to lower frequencies than the TM (Ehret *et al.* 1990, 1994). This constitutes evidence for interactions between vibrating structures having dissimilar resonance frequencies.

The noise levels used in this study are of low spectral densities that render difficult the detection of nonlinearities in the TM vibration velocity. However, these levels encompass the range of environmental noises to which these frogs are exposed in nature (Penna *et al.* 2005), and so the measurements conducted are biologically meaningful.

The invariance of the tympanic membrane response contrasts with the effect of noise at different stages of the anuran auditory pathway. Auditory fiber discharge rates and degree of phase locking in treefrogs and bullfrogs are altered by noise presented at 10–30 dB below the SPL of a tonal stimulus (Ehret and Capranica 1980; Hillery and Narins 1984; Narins 1987; Narins and Wagner 1989). Given that the middle-ear ossicles in the frog also behave linearly over at least a 20 dB dynamic range (Mason and Narins 2002), the first non-linear stage of the frog auditory pathway is likely to reside in the sensory hair cells in the amphibian and basilar papillae.

Responses of midbrain auditory neurons of ranid frogs are altered in similar ways as auditory nerve fibers under noise exposure, increasing their response thresholds (Ratnam and Feng 1998). However, under certain circumstances, midbrain auditory neurons show another kind of non-linearity; their responsiveness to stimuli is enhanced in the presence of broadband noise at low levels in terms of discharge rate (Ratnam and Feng 1998) or synchrony (Bibikov 2002). Such an effect is consistent with stochastic resonance in the auditory system. In contrast with these non-linear neural responses, in *E. calcaratus* a significant proportion (approximately 40%) of the midbrain auditory neurons does not decrease their discharge rates in response to tone bursts in the presence of increasing levels of noise. Rather, approximately half of these neurons maintain a constant discharge rate and a similar proportion increase their discharges at intermediate rates, decreasing them at higher rates (Penna *et al.* submitted). Such response patterns, (in particular neurons maintaining a constant discharge rate across the entire range of noise exposure levels) could result from the preservation of the immunity of the linearity of the response of the TM to tones under noise exposure reported here, along ascending levels in the auditory pathway.

Behavioral studies have shown important effects of broadband noise on the evoked vocal responses in a variety of frog species (Narins 1982, Gerhardt and Klump 1988; Schwartz and Gerhardt 1989; Narins *et al.* 1997; Penna *et al.* 2005; Sun and Narins 2005; Penna and Hamilton-West 2007). For males of *E. calcaratus*, broadcasting noise in their natural environment at levels between 66 and 78 dB SPL results in a significant increase in call rate and duration (Penna *et al.* 2005). Such persistence of evoked vocal responses is congruent with the existence of midbrain auditory neurons that preserve their responsiveness to sounds in the presence of accompanying noise and with a linear mechanical response of the eardrum preserving tone detection amid noise.

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Fig. 1. Waveforms (left column) and spectra (right column) of the sounds recorded with a microphone positioned above the head of a male of *Eupsophus calcaratus*. (a) stimulus waveforms and (b) spectra of recordings during intervals of presentation of a 1.5 kHz tone at 80 dB SPL in absence of generated noise and in the presence of band-pass noise at different levels. (c) Stimulus waveforms and (d) spectra of recordings during intervals of presentation of broadband noise. Spectra were obtained averaging over 100-ms waveform intervals. Noise levels are indicated on the right side of the graphs. No responses to the presentation of noise at 90 dB SPL are shown (see Materials and methods)

Fig. 2.

Waveforms (left column) and spectra (right column) of the laser vibrometer output for the same sounds presented in Fig. 1. (a) Stimulus waveforms and (b) spectra of recordings during intervals of presentation of a 1.5 kHz tone at 80 dB SPL in absence of generated noise and in the presence of band-pass noise at different levels. (c) Stimulus waveforms and (d) spectra of recordings during intervals of presentation of broadband noise. Spectra were obtained averaging over 100-ms waveform intervals. Noise levels are indicated on the right side of the graphs.

Fig. 3. Peak vibration velocity of the TM as a function of stimulus frequency for a representative male of *Eupsophus calcaratus* to pure tones at a constant level of 80 dB RMS SPL (a) in the absence of band-pass noise (upper graph) and in the presence of band-pass noise (b–f) at increasing intensities indicated on the right of each graph. (g) Vibration velocity measured in the same individual at the bony surface dorsal to the TM to the pure tones at a constant level of 80 dB RMS SPL in the absence of band-pass noise. Best frequency indicated with an arrow in (a) is the same for all graphs.

Fig. 4. TM velocity of the same individual as in Fig. 3 in response to pure tones of 1.5 kHz presented at intensities ranging from 50 to 104 dB SPL in 6-dB steps. Best-fit line for the data is shown.

Fig. 5.

(a) Pearson correlation coefficient for the TM vibration velocity in response to pure tones between intervals in the absence of generated noise and in the presence of subsequent presentations of different levels of band-pass noise. (b) Center frequency calculated from the area under the tuning curve considering only values within 20 dB below the maximum velocity in the absence and in the presence of band-pass noise at different intensities indicated in the abscissa. N: no band-pass noise. Circles and bars indicate averages and standard errors, respectively. Numbers above symbols indicate experimental subjects tested at each noise level.