Naturwissenschaften (2011) 98:1057–1061
DOI 10.1007/s00114-011-0851-7

SHORT COMMUNICATION

Tympanal mechanics and neural responses in the ears of a noctuid moth

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Received: 24 July 2011 / Revised: 22 September 2011 / Accepted: 26 September 2011 / Published online: 12 October 2011

Abstract Ears evolved in many groups of moths to detect the echolocation calls of predatory bats. Although the neurophysiology of bat detection has been intensively studied in moths for decades, the relationship between sound-induced movement of the noctuid tympanic membrane and action potentials in the auditory sensory cells (A1 and A2) has received little attention. Using laser Doppler vibrometry, we measured the velocity and displacement of the tympanum in response to pure tone pulses for moths that were intact or prepared for neural recording. When recording from the auditory nerve, the displacement of the tympanum at the neural threshold remained constant across frequencies, whereas velocity varied with frequency. This suggests that the key biophysical parameter for triggering action potentials in the sensory cells of noctuid moths is tympanum displacement, not velocity. The validity of studies on the neurophysiology of moth hearing rests on the assumption that the dissection and recording procedures do not affect the biomechanics of the ear. There were no consistent differences in tympanal velocity or displacement when moths were intact or prepared for neural recordings for sound levels close to neural threshold, indicating that this and other neurophysiological studies provide good estimates of what intact moths hear at threshold.

Keywords Moth auditory biomechanics · Neurophysiology · Auditory threshold · Lepidoptera

Communicated by: Sven Thatje

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Introduction

The neuroethology of predator detection and avoidance has been intensively studied in eared moths for more than five decades. Roeder and Treat (1957) provided the first evidence that some moths can hear the ultrasonic echolocation calls of bats, accounting for field observations of moth evasive flight in response to bats. Since then, physiological, evolutionary, and ecological aspects of this predator–prey relationship have been studied in the context of the auditory capabilities of moths, often using moths of the family Noctuidae (Fullard 1998; Miller and Surlykke 2001; Waters 2003). The two sensory cells (A1 and A2) of the noctuid moth ear have different thresholds for sound and are directly attached to the tympanum at the location of maximal tympanal displacement (Windmill et al. 2007). Despite detailed knowledge of the morphology of these ears and the physiology of the two sensory cells, the mechanism of transduction from tympanal vibrations to action potentials is unknown (Yack 2004).

Our objective was to investigate the relevant input to this transduction process by determining which motion property of the tympanum (velocity or displacement) elicits neural activity in the sensory A-cells. The tympanal motion property that elicits neural activity will remain constant at neural threshold across frequencies. The other motion property will change with frequency due to the interdepen-

dence of the parameters velocity, displacement, and frequency (velocity is the derivative of displacement with respect to time, i.e., the inverse of frequency). For example, to maintain a constant displacement, the tympanum must move with greater velocity at higher frequencies. To record from the auditory nerve of noctuid moths, the thorax is dissected open, many of the flight muscles are removed, and the nerve is hooked with an extracellular electrode (Roeder 1966). As this procedure might modify tympanal responses, we also tested the assumption that the tympanum behaves the same in intact and dissected moths.

Materials and methods

Moths (*Noctua pronuba*) were collected with light traps in and around Bristol, UK, and used within 24 h. Moths were immobilized with their dorsal side against modelling clay with the wings pinned to the sides. Scales surrounding one ear were removed, and the laser beam of a microscanning laser Doppler vibrometer (PSV-300-F with OFV-056 scanning head and close-up attachment, Scanning Vibrometer software 7.4; Polytec GmBH, Waldbronn, Germany) was positioned at the sensory cells' attachment site on the tympanum. A loudspeaker (ScanSpeak Model 60102, Avisoft Bioacoustics, Berlin, Germany) was directed at the moth's ear from a 30-cm distance. A 1/8" microphone (type 4138, preamplifier type 2633, Brüel and Kjær, Nærum, Denmark) measured the incident sound pressure level (SPL; decibels (dB) re 20 μPa) ca. 2 mm beside the moth's ear. All equipment and the moth were placed on a vibration isolation table (TMC 784-443-12R; Technical Manufacturing Corp., Peabody, MA, USA) inside an acoustic isolation booth (IAC series 1204A; Industrial Acoustics, Bronx, NY, USA) with an average room temperature of 22–23°C and relative humidity of 50–60%.

Acoustic and vibrational measurements were recorded (204.8 kHz sampling frequency) from each moth in three sequential treatments: (1) intact moth with the ventral side facing down (intact, down: the natural flight position), (2) intact moth with the ventral side facing up (intact, up), and (3) dissected moth with electrodes inserted and the ventral side facing up (electrode). The starting treatment for each moth alternated between 1 and 2 to reduce any effect of order. Sound stimuli were series of 20 ms tone-pulses (plus raised-cosine ramps of 2 ms) at a period of 200 ms, increasing in 5 dB steps from an expected value of 25 to 90 dB SPL (based on free-field speaker calibration with only the microphone present). Pulse-series at 16 frequencies (5 to 80 kHz in 5-kHz increments) were presented once in random order using Avisoft Bioacoustics Recorder software via a data acquisition board (USB-6251, National Instruments, Austin, TX, USA). We based our analysis on measured incident SPL. Incident SPL deviated from the expected SPL by −5.4 dB on average (N=6,356), likely due to different reflection and interference patterns caused by the added equipment compared with calibration and despite echo-attenuating all hard surfaces surrounding the moth.

To obtain tympanal displacement, the velocity recording was integrated. Then, using the microphone and vibrometer recordings, we calculated the root mean square (RMS) of the incident SPL and the elicited tympanal velocity level (VL) and displacement level (DL). SPL (dB re 20 μPa), VL (dB re 50 nm/s, VL=20×log10 (velocity/50 nm/s)) and DL (dB re 100 pm, DL=20×log10 (displacement/100 pm)) were calculated in two steps for each pulse in a series. First, the RMS of each pulse was calculated, and values below twice the background noise RMS were excluded from further analysis. Second, in order to obtain RMS-values for those excluded pulses, we cross-correlated a pulse template with the recordings, calculated the Hilbert-envelope of the cross-correlation function (CCFE) and obtained the peak-amplitude of the CCFE for each pulse. CCFE peak-amplitudes below three times the maximum of the background noise CCFE were excluded. The RMS-values of the pulses that had been excluded in step 1 were obtained by relating the CCFE peak-amplitudes of the excluded pulses to the CCFE peak-amplitudes of pulses with direct RMS measurements. For the SPL, we additionally calculated linear regressions between expected and incident SPL (R^2=1 for all) and used the calculated SPL values of the linear regression for all pulses, including extrapolating to those whose CCFE peak-amplitude was below threshold. We used second-order polynomial regressions to describe the relationship between incident SPL and elicited VL and DL (accounting for the plateau at high SPL in the otherwise linear relationships), excluding regressions with R^2<0.9. We compared tympanal movements (VL and DL) between treatments at various SPLs between 30 and 80 dB SPL.

During the third treatment, we recorded the activity of the A1 and A2 sensory cells simultaneously with tympanal vibrations. Moths remained pinned to modelling clay while removing ventral sternites and large thoracic flight muscles to expose the auditory nerve, which was hooked with an extracellular electrode (Roeder 1966). An indifferent electrode was placed in the abdomen. Electrode signals were amplified (custom-built amplifier) and recorded (National Instruments USB-6251, Avisoft Recorder software). To determine the adequate stimulus for the sensory cells, we obtained audiograms that show threshold SPL, VL, or DL as a function of frequency. The SPL at neural threshold for each sensory cell is the lowest amplitude sound pulse that consistently elicited one or more action potentials (maximum latency set to 25 ms, defined as the time from when the tympanum started to vibrate to the peak of the first action potential). Likewise, the tympanal VL and DL at neural threshold were defined as the VL and DL

measured for that sound pulse. We additionally obtained SPL, VL, and DL using a first A1 spike latency of 8 ms as threshold and also calculated the latency audiograms at constant VL and DL to describe A1 temporal characteristics.

We measured ten moths, but the final sample size differs between treatment and statistical test depending on technical failures, recording noise, and regressions with $R^2<0.9$. Data were analyzed with general linear mixed models (SPSS 15, IMB, Armonk, NY, USA). For the treatment comparison, we calculated separate models for each frequency, using individuals as random and treatment as repeated fixed factor. Models to determine the adequate stimulus included individual as random factor and frequency as repeated covariate.

Results

At low sound pressure levels, there were few significant differences between VLs or DLs at a given frequency for the three different treatments (Fig. 1). There was also no consistent pattern between treatments; the mean VL and DL during neural recordings was higher than, lower than, or intermediate to the two intact recordings depending on frequency. Likewise, post hoc pairwise comparisons revealed that not all the differences were between the electrode and both intact treatments, but also between the electrode and only one intact or between both intact treatments. As sound level increased, however, tympanal

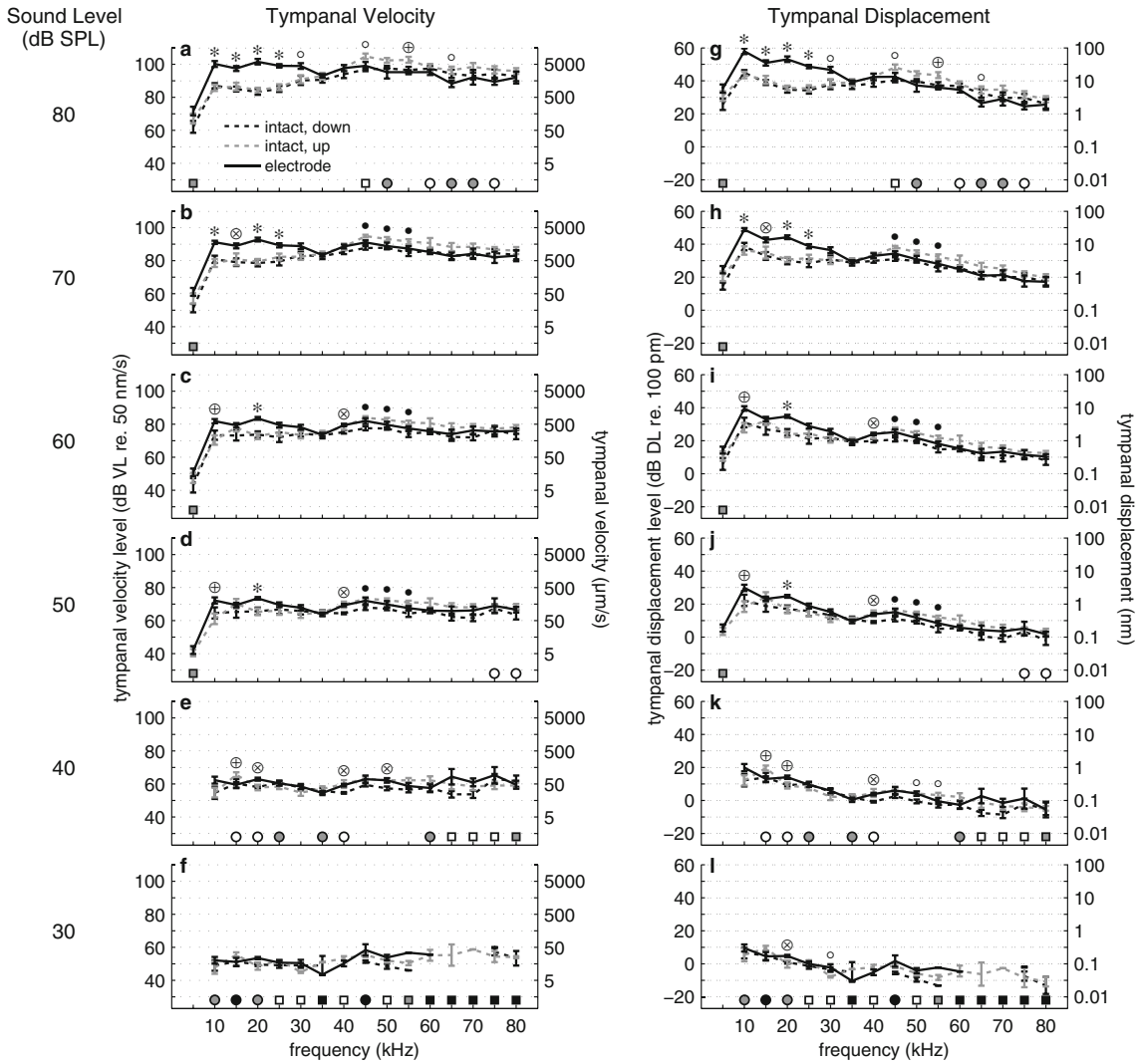

Fig. 1 Treatment comparisons: Tympanal velocity (**a**–**f**) and displacement (**g**–**l**) elicited by sound pulses of different sound pressure levels for three experimental treatments. Mean ± SEM of one to ten moths. *Symbols above curves* indicate significant ($p<0.05$) overall treatment effects. *Symbol type* indicates which subsequent post hoc pairwise comparisons were significant (Bonferroni-corrected): ○: no significant pairwise comparisons, •: Down vs. Up, ⊗: Down vs. Electrode, ⊕: Up vs. Electrode, *asterisk*: both intact (down and up) vs. Electrode. *Symbols* at *bottom* of *axes* indicate the smallest sample size of all three treatments. No symbol: N=7–10 moths for all three treatments; *circle*: N=4–6 moths for one (*open*), two (*grey*), or all three (*black*) treatments, *square*: N=0–3 moths for one (*open*), two (*grey*), or all three (*black*) treatments

VL and DL at lower frequencies (<35 kHz) became increasingly greater for the electrode treatment than the intact treatments (Fig. 1).

Given that neurophysiological methods did not affect tympanal vibration at low sound levels such as those at neural threshold, we set out to determine the nature of the adequate stimulus for the sensory A-cells. SPL audiograms at neural threshold (Fig. 2a) were typical for those previously reported for *N. pronuba* (Waters and Jones 1996; Tougaard 1998). Neural VL and DL audiograms differed from each other (Fig. 2b, c). When excluding 5 and 10 kHz, the slopes of linear regressions over frequency decreased from SPL, to VL, to DL, and while SPL and VL increased with frequency, DL was stable across frequencies. The mean displacement at neural threshold from 15 to 80 kHz was 140 (±7 SEM) pm for A1 and 2510 (±180 SEM) pm for A2. The same pattern was found between frequency and SPL, VL, and DL using a first spike latency of 8 ms as threshold (Fig. 2d–f). When the A1 cell was stimulated by a constant tympanal velocity, its first spike latency increased with frequency (Fig. 2g), but it varied only slightly around 7 ms at a constant DL (Fig. 2h).

Discussion

Our data demonstrate that, for pure tone pulses and at low SPLs, tympanal movement is not systematically affected by ventral dissection and hooking of the auditory nerve used to record from the sensory cells of noctuid moths. Therefore, previous studies on moth hearing thresholds (audiograms) are valid estimates of what intact moths hear. Many bat species produce narrowband or constant frequency calls (Fenton 1990). For these bat species, audiograms, when corrected for signal duration, provide reasonable estimates of hearing thresholds for bat calls. Our data cannot assess, however, how the ear might behave in response to frequency-modulated sweeps, which are typical for many other bat species (Fenton 1990). In addition, neuronal data obtained at low frequencies and at SPLs much greater than threshold should be interpreted with caution due to differences in the movement of the tympanum between intact and dissected moths.

Two lines of evidence suggest that the displacement of the tympanum, not its velocity, is the adequate stimulus for the sensory cells: (1) the displacement is constant both at neural threshold and at 8 ms A1-first spike latency across all frequencies (except 5 and 10 kHz), whereas velocity increases with frequency, and (2) the first spike latency of the A1 cell is the same across all frequencies (except 5 and 10 kHz) at a constant displacement, but increases with frequency at a constant velocity. Previous estimates of the minimum displacement detected by the A1 cell are 10–100 pm (Adams 1972) and 200 pm (Windmill et al. 2007), which agree with our measurement of 140 (±7 SEM) pm.

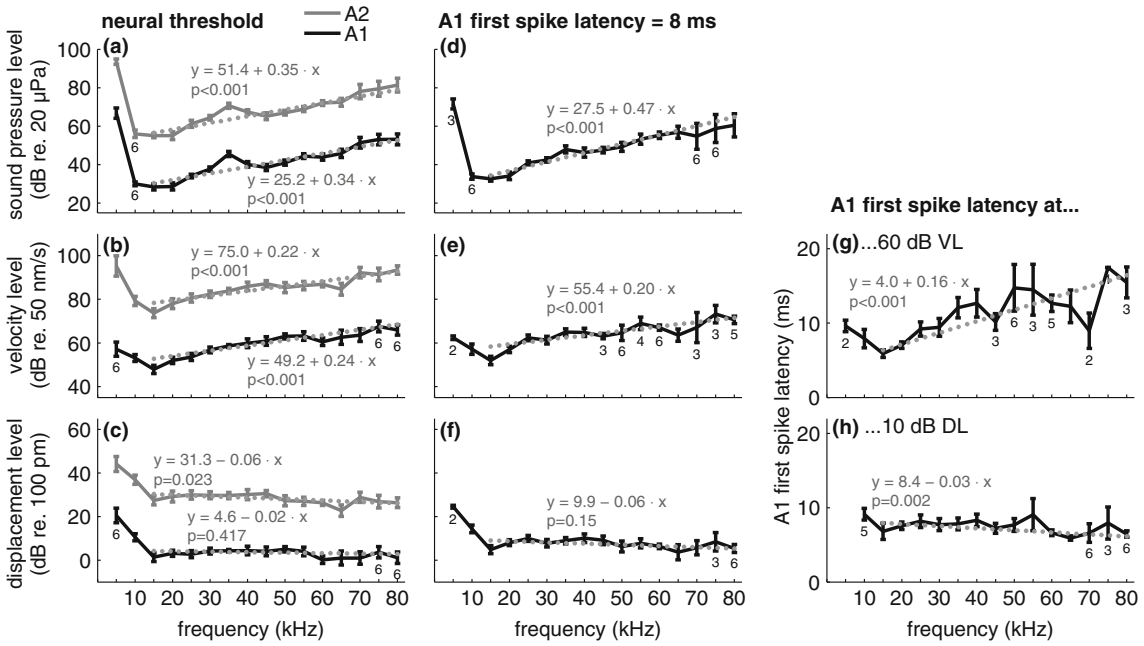

Fig. 2 Adequate stimulus: Audiograms (**a**–**f**) show the mean RMS sound pressure (**a**, **d**), tympanal velocity (**b**, **e**) or tympanal displacement (**c**, **f**) level required to elicit a specific neural response. Response was defined as neural threshold of the A1 (*black*) and A2 (*grey*) cells (**a**–**c**) and as a first spike latency of 8 ms of the A1 cell (**d**-**f**). A1 temporal characteristics (**g**–**h**): A1 first spike latency measured at a constant VL (**g**) and DL (**h**). Mean ± SEM of one to nine moths. *Small numbers* are the sample size if $N<7$ moths

Two steps in the hearing process contribute to poor low-frequency hearing in noctuid moths: coupling of sound energy to tympanal movement and transduction of tympanal movement to action potentials by neurons. By comparing neural thresholds in response to natural sound stimulation and in response to direct mechanical stimulation of the tympanum, Adams (1972) concluded that the coupling of sound energy to tympanal movement is poor at frequencies below 10 kHz in a noctuid moth. Our data also support this conclusion (Fig. 1). In contrast to Adams (1972), who found differences in displacement across frequencies at neural threshold using mechanical stimulation, we measured constant displacements at neural threshold for frequencies of 15 kHz and above, using sound, the natural stimulus. The difference in our results is likely due to the difference in method. We found that displacement at neural threshold is greater for 5 and 10 kHz, meaning that the ability of the neurons to transduce tympanal movement to action potentials also decreases at lower frequencies. Therefore, low-frequency sounds are filtered out at both steps in the hearing process, which tunes moth ears to frequencies of biological importance, the echolocation calls of bats. Our data show that the key biophysical parameter for triggering action potentials in the sensory cells of moths is the displacement of the tympanum. In addition, we validate previous neurophysiological hearing thresholds obtained from moths using these methods, which provide valuable insight into the sensory ecology of predator–prey interactions and suggest using caution when interpreting neurophysiological data collected at higher sound levels.

Acknowledgments This study was supported by the Biotechnology and Biological Sciences Research Council (BB/f002386/1, MWH), a HFSP Cross Disciplinary Fellowship (LT00024/2008-C, FM-Z) and the Royal Society of London (DR). We thank J. Memmott and G. Jones for collecting moths, A. Radford and I. Cuthill for statistical advice, and B. Hedwig, F. Dupuy, and two anonymous reviewers for comments on earlier versions of this manuscript.

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