Tiger moths and the threat of bats: decision-making based on the activity of a single sensory neuron

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Echolocating bats and eared moths are a model system of predator–prey interaction within an almost exclusively auditory world. Through selective pressures from aerial-hawking bats, noctuoid moths have evolved simple ears that contain one to two auditory neurons and function to detect bat echolocation calls and initiate defensive flight behaviours. Among these moths, some chemically defended and mimetic tiger moths also produce ultrasonic clicks in response to bat echolocation calls; these defensive signals are effective warning signals and may interfere with bats' ability to process echoic information. Here, we demonstrate that the activity of a single auditory neuron (the A1 cell) provides sufficient information for the toxic dogbane tiger moth, *Cycnia tenera*, to decide when to initiate defensive sound production in the face of bats. Thus, despite previous suggestions to the contrary, these moths' only other auditory neuron, the less sensitive A2 cell, is not necessary for initiating sound production. However, we found a positive linear relationship between combined A1 and A2 activity and the number of clicks the dogbane tiger moth produces.

Keywords: antipredator behaviour; neuroethology; sensory ecology; cognitive ecology

1. INTRODUCTION

Echolocating bats possess exceptionally sophisticated auditory systems (Popper & Fay 1995); at the other end of the spectrum, noctuoid moths' tone-deaf ears each contain two to three neurons (Fullard *et al.* 2003). However, owing to spherical spreading and atmospheric attenuation of sound, eared moths detect bats' echolocation calls at distances greater than bats detect moths (Surlykke 1988). In noctuid and arctiid moths, two of these neurons are auditory afferents (A1 and A2; A1 is the more sensitive of the two cells); the third (the B cell) appears to be a proprioceptor, exhibiting no response to sounds (Fullard *et al.* 2003). Echolocation call-evoked A1 activity occurs at distances before the bat has detected the

moth. Confronted with an attacking bat, the bat's echolocation calls elicit A2 activity (Roeder 1967; Fullard *et al.* 2003). Roeder (1974) proposed that evasive behaviours of noctuid moths are bimodal and negative phonotaxis is initiated by A1 activity and erratic flight by A2 activity. However, another group of noctuoids, the notodontids, exhibit a bimodal response to distant versus proximate bats but possess no A2 cell. Thus, in the absence of the A2 cell, echolocation call-induced changes in A1 cell spike number, rate or both appear sufficient for initiating both kinds of flight (Surlykke 1984). A more recent study has suggested that A2 is unimportant in evoking erratic flight in noctuoid moths in general (Fullard *et al.* 2003).

When stimulated by intense ultrasound the dogbane tiger moth, *Cycnia tenera*, produces bursts of roughly 14 ultrasonic clicks (modulation cycles (MCs), mean cycle length of approximately 18 ms) from each of two thoracic tymbals (Blest *et al.* 1963). Clicks are an effective defence against attacking bats (Ratcliffe & Fullard 2005). In *C. tenera*, one explanation for the existence of A2 is that it is necessary for initiating sound production (Fullard 1992; Fullard *et al.* 2003). These studies used short-duration (less than 10 ms) pulses and A2 activity was consistently observed to precede phonoresponse (Fullard 1992; Dawson & Fullard 1995; Fullard *et al.* 2003). To test the alternative hypothesis that A2 is not necessary for initiating defensive sound production in response to bat echolocation calls, we used longer pulses of short and long rise times to effect differences in A1 and A2 activity over a range of maximum sound pressure levels. Short rise times were used to replicate rise times used in previous studies; long rise times to better simulate echolocation calls at moths' ears. While intuitively appealing, Roeder's (1974) bimodal hypothesis has, thus far, proved impossible to test because of variability in flight responses. Here, we extrapolate our results from defensive sound production to anti-bat evasive flight and offer a plausible alternative hypothesis for its bimodal nature.

2. MATERIAL AND METHODS

(a) *Animals and acoustic presentation*

Experiments were conducted at Queen's University Biological Station (QUBS) in southeastern Ontario, Canada. *Cycnia tenera* eggs were taken from wild-caught adults and raised to pupae on dogbane, *Apocynum androsaemifolium*, and Indian hemp, *Apocynum cannabinum*. Pupae were stored at 4°C (12 L : 12 D cycle) for several months, and then transferred to 25°C (16 L : 8 D) rooms. Adults emerged two to three weeks later and matured for 12–24 hours. Moths were exposed to pulsed synthetic sounds generated by MATLAB (v. R2006b, MathWorks, USA), broadcast via a high-speed data acquisition card (National Instruments, Austin, TX, USA), ultrasonic amplifier (70101, Avisoft Bioacoustics, Germany) and ultrasonic speaker (ScanSpeak 60102, Avisoft). The speaker was 20 cm behind and ventral to the moth in the chamber (*behaviour*) and foam-lined Faraday cage (*electrophysiology*). This system was calibrated and intensities were measured as described in Fullard *et al.* (2003).

(b) *Behaviour*

Moths were tethered from their dorsal thorax using wax and a rigid wire, suspended in a foam-lined chamber and left in darkness for 20 min before playbacks began. After acclimatization, moths remained relatively motionless throughout trials. Acoustic stimuli and tymbal MCs produced in response to these stimuli were detected using an Avisoft CM16 microphone and recorded using an ultrasound acquisition board (Avisoft USG 416) connected to a laptop running AVISOFT RECORDER at a sampling rate of 250 kHz. The .wav files were subsequently analysed using BATSOUND PRO v. 3.2 (Pettersson Elektronik, Sweden). The microphone was
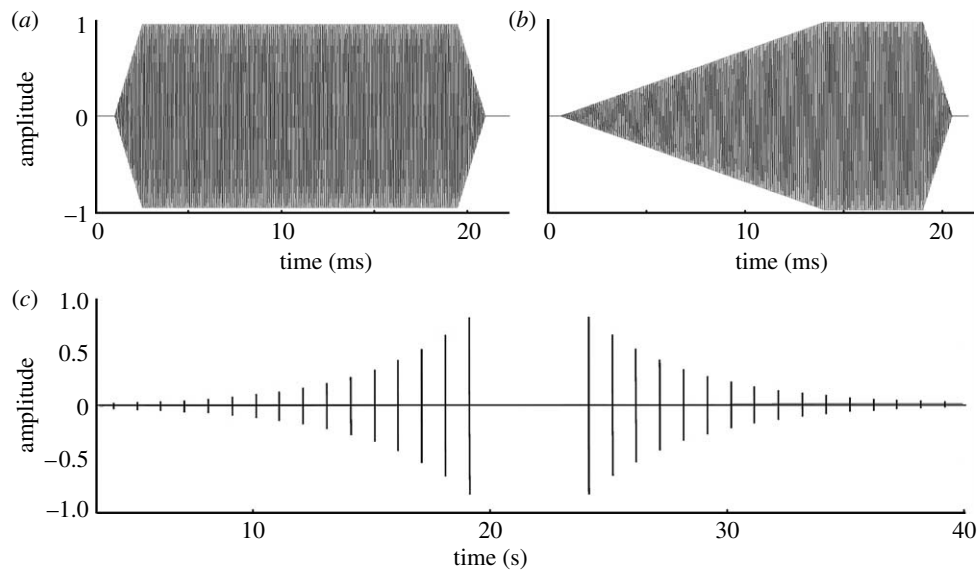
Figure 1. All pulses were 20 ms, 40 kHz tones. (*a*) Short-rise pulses had 1.5 ms rise/fall times; (*b*) long-rise pulses had 13.5 ms rise times and 1.5 ms fall times. (*c*) Each series consisted of 16 short- or long-rise pulses of ascending intensity (70–100 dB peSPL @ 2 dB increments; pulse period = 1 s), 5 s of silence, followed by 16 pulses of the same design descending in intensity.

Table 1. Neural activity and sound production data for five moths tested. (Behavioural data for each moth while both ears were intact (*i*) and after one ear was ablated (*a*); $^*p < 0.05$; circles indicate a negative slope value. A1 minimum inter-spike period (minISP) was always lower than 2.6 ms, the minISP suggested by Roeder (1967) as required for initiating evasive flight and was an unreliable predictor of initial phonoreponse and MC number.)

	threshold values of initial phonoresponse (short-rise/long-rise pulses)				click modulation cycle regressions, adj. r^2 (short-rise/long-rise pulses)		
moth	dB	A1 minISP	no. of MCs	A1(A2) spikes	A1 minISP	A1 spike no.	A1+A2 spike no.
1 (*i*)	72/74	1.8/1.7	1/1	9(0)/10(0)	0.57°/0.60°	0.84*/0.69*	0.95*/0.86*
1 (*a*)	84/86	1.3/1.2	1/1	17(7)/14(8)	0.26°/0.29°	0.51*/0.43*	0.78*/0.61*
2 (*i*)	74/74	1.3/1.2	4/1	8(0)/7(0)	0.22°/0.22°	0.63*/0.84*	0.64*/0.69*
2 (*a*)	78/	1.2/	1/	16(0)/	0.00/	0.41*/	0.08/
3 (*i*)	72/76	1.4/1.4	1/1	16(0)/15(0)	0.08°/0.26°	0.28*/0.66*	0.28*/0.66*
3 (*a*)	76/80	1.4/1.4	1/1	20(0)/17(0)	0.00/0.75*	0.19/0.88*	0.19/0.88*
4 (*i*)	72/74	1.4/1.4	1/5	17(0)/14(0)	0.00/0.03°	0.09/0.41*	0.63*/0.75*
4 (*a*)	82/	1.4/	1/	16(14)/	0.00/	0.00/	0.04/
5 (*i*)	72/74	1.3/1.4	1/1	18(0)/16(0)	0.00/0.45*	0.23*/0.57*	0.80*/0.93*
5 (*a*)	80/82	1.2/1.2	2/1	20(11)/17(9)	0.02/0.43°	0.30°/0.45*	0.62*/0.74*

equidistant from the moth and the speaker. We noted the total number of MCs produced to each pulse. Moths were exposed to 32 short-rise pulse, 32 long-rise pulses and then 32 short-rise pulses once more (each series 10 s apart; total duration 145 s; figure 1). We then ablated one ear and re-exposed each moth to the stimulus paradigm to later determine how many afferent spikes would have been necessary to elicit the observed number of click MCs in monaural versus binaural subjects prior to electrophysiological preparation.

(c) Electrophysiology
Approximately 30 min after behavioural trials, we prepared the same moths for extracellular electrophysiology. Ablation does not affect the activity of the primary sensory neurons in the intact ear (Roeder 1967) and physiological data can thus be compared with binaural and monaural behavioural data. We used standard techniques (Fullard et al. 1998) to expose the auditory nerve (IIIN1b) and recorded action potentials in response to acoustic stimuli with a stainless steel hook electrode referenced to another in the abdomen (motor nerve activity recorded passively). Moths were not decapitated to prevent reduction in tymbal activation (Dawson & Fullard 1995). Responses were amplified (P15, Grass Instruments, Astro-Med, Warwick, RI USA), digitized at a 250 kHz sampling rate (TL2, Axon Instruments, Foster City, CA USA) and analysed using custom MATLAB applications.

Both behavioural and physiological data were averaged for each pulse design to control for ordering effects and it is these mean data that appear in table 1. Statistics are reported as mean ± 1 s.d.

3. RESULTS
For all moths tested, sound production in intact moths first occurred at stimulus intensities that evoked no A2 activity during subsequent neural examinations (figure 2; table 1). Maximum pulse intensity at first response did not differ significantly between pulse designs (Wilcoxon signed-rank test, $n = 5$, $p > 0.05$). However, threshold A1 spike number per pulse was significantly lower for long-rise pulses than for short-rise pulses (Wilcoxon, $n = 5$, $p < 0.05$) while the number of MCs was significantly greater for short-rise pulses than for long-rise pulses (Wilcoxon, $n = 5$, $p < 0.05$). For a given pulse duration, this suggests that maximum intensity predicts the occurrence of a phonoresponse, but the
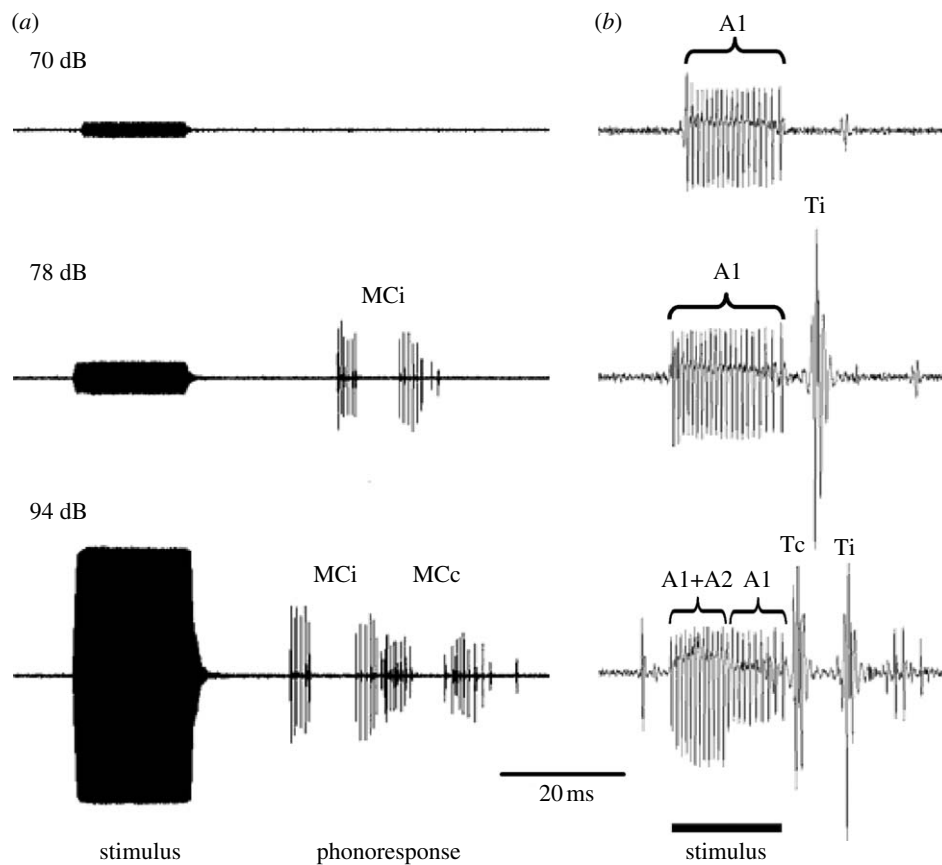
Figure 2. (*a*) *Cycnia tenera* phonoresponse (post-ablation) and (*b*) the neural activity that evokes it (data from moth 2 in table 1). A 70 dB pulse evokes no phonoresponse and an auditory response consisting of only A1 cell activity. A 78 dB pulse evokes a single MC on the ipsilateral side of the moth (MCi), a compound action potential from the ipsilateral tymbal motor nerve (Ti), but again only A1 spikes. A 94 dB pulse evokes MCs from ipsilateral (MCi) and contralateral (MCc) tymbals which are temporally separated due to the cyclical nature of the pattern generator that evokes them (Fullard 1992), compound action potentials from ipsilateral (Ti) and contralateral (Tc) tymbal motor nerves, and spikes from A1 and A2 cells (partially summated). Note that A1 and A2 receptor spikes are easily distinguished (Roeder 1967), with three conditions existing: (i) single unit activity of either A1 or A2, identified by their different extracellular spike amplitudes, (ii) partial spike summation, identified by the presence of multiple spikes within the same envelope, and (iii) complete spike summation, identified as a single spike whose amplitude is the sum of the two single spikes.

total energy of a given pulse the number of MCs. Regression analyses showed that the number of clicks was positively correlated with A1 activity in four out of five instances (table 1; see the electronic supplementary material). For short-rise pulses, A2 activity was only observed at and above intensities of 76–100 dB peak equivalent sound pressure level (peSPL; Stapells *et al.* 1982). For long-rise pulses, in four moths, A2 activity was observed at intensities at and above 78–84 dB peSPL. Long-rise pulses never elicited A2 activity in one of the moths tested (moth 3, table 1).

After ablating one ear, the phonoresponse to a given pulse intensity and design was significantly reduced compared with that prior to ablation (Wilcoxon, $n=20$, $p<0.05$). Post-ablation, moths continued to produce MCs by buckling their ipsilateral and contralateral tymbals (Dawson & Fullard 1995). All responded to short-rise pulses, three of five only after onset of A2 activity. Three moths responded to long-rise pulses, two only after onset of A2 activity (table 1).

For eliciting the first phonoresponse to short-rise pulses, the total number of A1+A2 cell spikes was roughly equivalent for moths before ((13.6 ± 4.7 A1 spikes $+0$ A2 spikes)$\times2$ ears ≈27 A1+A2 cell spikes) and after ablation (24.2 ± 6.4 A1+A2 spikes).

Slopes and adjusted r^2 values were significantly greater for A1+A2 spike number versus MC number regression analyses than for those performed using A1 spike number (two paired *t*-tests, $n=18$, $p<0.05$ for both tests; table 1; see the electronic supplementary material).

4. DISCUSSION

Previous studies have consistently shown A2 activity preceding sound production (Fullard 1992; Dawson & Fullard 1995; Fullard *et al.* 2003) and A2 activity was therefore assumed necessary for initiating this behaviour (Fullard 1992; Fullard *et al.* 2003). However, our physiological and matched behavioural data indicate that acoustically evoked A1 cell activity alone can initiate *C. tenera*'s phonoreponse (figure 2). These results allow us to reject the hypothesis that A2 activity is necessary for eliciting the phonoresponse under acoustic stimulation, and show instead that A1 activity is sufficient under these same conditions. However, we do not mean to suggest that the A2 cell plays no role in anti-bat sound production. Instead, our observation that monaural preparations require higher stimulus intensities to initiate defensive sound

production suggests that an auditory or tymbal command interneuron or interneural network integrates total A1 and A2 spike input from both ears, before engaging the tymbal central pattern generator. Indeed, the total number of A1+A2 cell spikes (for a single ear) predicts the initial phonoresponse and is positively and significantly related to MC number per pulse, whether the two ears are intact or one is ablated (table 1; see the electronic supplementary material).

Accordingly, the A2 cell should be interpreted as serving a functional role in the defensive behaviour of at least this species of noctuoid moth. Input from each of the moth's four A cells appears to be equivalent and additive at the central nervous system level. Noctuoid species with both cell types have more sensitive ears and fly more than one auditory-celled notodontids (ter Hofstede *et al.* 2008); for noctuid moths endemic to the bat-free habitat of Tahiti, A2 has degenerated more than A1 (Fullard *et al.* 2007). Similarly, maintenance of sound production in tiger moths over evolutionary time reflects species-specific exposure to bats (Ratcliffe & Nydam 2008). Because the A2 cell is not necessary for, but involved in, sound production, A2 may simply act as another, albeit less sensitive, A1 cell. Consequently, we speculate that the bimodal nature of anti-bat flight behaviour (Roeder 1967; Surlykke 1984) may represent a two-threshold mechanism based on the total A1+A2 cell spike number in noctuoid moths and propose this as an alternative to Roeder's (1974) hypothesis that A1 initiates negative phonotaxis and A2 erratic flight.

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