



Vocalization-induced middle ear muscle reflex and auditory fovea do not contribute to the unimpaired auditory sensitivity after intense noise exposure in the CF-FM bat, *Hipposideros pratti*

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Abstract

Behaviors and auditory physiological responses of some species of echolocating bats remain unaffected after exposure to intense noise, but information on the underlying mechanisms remains limited. Here, we studied whether the vocalization-induced middle ear muscle (MEM) contractions (MEM reflex) and auditory fovea contributed to the unimpaired auditory sensitivity of constant frequency-frequency modulation (CF-FM) bats after exposure to broad-band intense noise. The vocalizations of the CF-FM bat, *Hipposideros pratti*, were inhibited through anesthesia to eliminate the vocalization-induced MEM reflex. First, the anesthetized bats were exposed to intense broad-band noise, and the findings showed that the bats could still maintain their auditory sensitivities. However, auditory sensitivities were seriously impaired in CBA/Ca mice exposed to intense noise under anesthesia. This indicated that the unimpaired auditory sensitivity in *H. pratti* after exposure to intense noise under anesthesia was not due to anesthetization. The bats were further exposed to low-frequency band-limited noise, whose passband did not overlap with echolocation call frequencies. The results showed that the auditory responses to sound frequencies within the noise spectrum and one-half octave higher than the spectrum were also unimpaired. Taken together, the results indicate that both vocalization-induced MEM reflex and auditory fovea do not contribute to the unimpaired auditory sensitivity in *H. pratti* after exposure to intense noise. The possible mechanisms underlying the unimpaired auditory sensitivity after echolocating bats were exposed to intense noise are discussed.

Keywords Echolocation · Middle ear muscle · Auditory fovea · Noise susceptibility · Hearing loss

Introduction

Most animals exhibit elevated auditory thresholds after being over-exposed to intense sounds, which is a well-known phenomenon called noise-induced hearing loss (NIHL). The severity of NIHL varies among animal species, though mammals are thought to be most sensitive to

noise exposure (Rubel et al. 2013). However, recent studies showed that behaviors and auditory physiological responses of some species of echolocating bats, which are the only true flying mammals on Earth, remain unaffected after exposure to intense noise (Simmons et al. 2015, 2016, 2017, 2018; Hom et al. 2016). A comparative study among echolocating bats, nonecholocating fruit bats, and laboratory mice revealed that the cochlear hair cell protecting gene *ISL1* was overexpressed in echolocating bats. It was also shown that transfecting this gene into the organ of Corti explant could partially rescue the explant from ototoxic drugs. This suggested that the overexpression of *ISL1* might contribute to the unimpaired auditory sensitivity toward intense noise in echolocating bats (Liu et al. 2021).

Echolocating bats are classified into constant frequency-frequency modulation (CF-FM) bats, FM bats, and click bats based on the spectral properties of their echolocation

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signals. The CF-FM bats have relatively long pulse durations compared to FM bats and click bats, which makes them call at high duty cycles of up to 80% (Jones 1999). Considering that the middle ear muscle (MEM) contracts before vocalization and relaxes after vocalization (MEM reflex), high duty-cycles induce long-lasting contraction time for the MEM, which can reduce about 20–30 dB SPL of sound (Pollak and Henson 1973; Jen and Suga 1976). In FM bats, vocalization-induced MEM reflex may have a negligible contribution to protecting bats from intense noise (Simmons et al. 2016), considering that the duty cycles are usually less than 7% (Jones 1999). However, whether the vocalization-induced MEM reflex has a role to play in the unimpaired auditory sensitivity of echolocating bats after exposure to intense noise in high-duty-cycle CF-FM bats remain an open question.

The echolocation signals of CF-FM bats usually consist of 3–4 harmonics (H1–H4), and their peripheral auditory systems are highly specialized for fine frequency analysis of the CF component of H2 (CF2) and detecting echo CF2 from fluttering insects, which is based on the auditory fovea in their cochleae (Suga 2018). Therefore, neurons in auditory fovea in the cochleae and foveal areas in higher auditory centers have extremely sharp frequency tuning curve (Schnitzler and Denzinger 2011). Hence, if the spectrum of noise spans a wide range, the sharp frequency tuning makes its passband overlap little with the noise spectrum and thus makes the noise less effective. Thus, the auditory fovea may also contribute to the unimpaired auditory sensitivity in CF-FM bats after exposure to broad-band intense noise.

In the present study, the electrophysiological data of auditory sensitivity before and after the CF-FM bat (*Hipposideros pratti*) was exposed to intense noise and that from a mouse model (CBA/Ca) were combined. The study reports that both vocalization-induced MEM reflex and auditory fovea do not contribute to unimpaired auditory sensitivity after intense noise exposure in the CF-FM bat, *H. pratti*.

Methods and materials

Ethical approval

The study was approved by the Institutional Animal Care and Use Committee of Central China Normal University, Wuhan, Hubei, PRC (Permit Number: CCNU-IACUC-2022-011). The Forestry Department of Hubei Province provided permission to conduct the study. All surgeries and recordings were performed under anesthesia with a mixture of Nembutal (40 mg/kg) and Xylazine (5 mg/kg).

Animals and housing

Fifteen *H. pratti* (ten males, five females; 47.3–75.0 g body weight) and 24 CBA/Ca mice (all males; 20.3 ± 0.26 g) were used in this study. The bats were collected from a cave (N: 29°26′0.32″; E: 114°01′20.49″) near Xianning City of Hubei province, China. The bats were wild caught using a hand net, during the daytime. All bats were housed in an animal room (dimensions: 3.0 m × 3.0 m × 3.0 m) and were exposed to the local photoperiod with constant temperature (28–30°C) and humidity (> 60%). The bats had free access to water and food (mealworms). They were examined daily for any signs of weakness, including an empty stomach or slow response to being handheld. Bats that were observed to be in poor physiological condition were excluded from that day's experiment and returned to the animal room. The Forestry Department of Hubei Province provided permission to conduct the study.

Six-week-old CBA/Ca mice were purchased from Nanjing Junke Bioengineering Co., Ltd (Nanjing, China). All mice had free access to sterile water and commercial food (Hubei Experimental Animal Center). They were acclimated in specific pathogen free (SPF) conditions for two weeks before experimental measurements were taken at a temperature of 24–26 °C, relative humidity of 55–75%, and a standard 12:12 h light-dark cycle. The mice remained housed in these conditions for the entire experimental period in the animal facility of the School of Life Sciences at Central China Normal University.

Recordings of echolocation pulses

Bats' echolocation pulse recordings were taken before each electrophysiological measurement. This was done to quantify the frequency spectrum for each bat's echolocation pulse. During the recordings, each bat hung on the ceiling of the experimental anechoic room and the recording microphone was placed 1 m below the bat in its frontal azimuth space. Real-time recordings of the echolocation pulses were recorded from each sedentary bat using a handheld ultrasound detector (Petterson D1000X, Pettersson Elektronik AB, Sweden) before they were entered into a notebook computer. Each bat was recorded one to three times, and each lasting between 3 and 5 s.

Pulse analysis

The echolocation pulses were analyzed using the BatSound pro 3.31b software (Pettersson Elektronik AB, Sweden), with a fast Fourier transformation (FFT) size of 1024 points and a Hanning window, using a cursor and visual

determination on a screen. The sound intensities were collected from the spectrograms.

Noise exposure

The bats were anesthetized using a mixture of Nembutal (40 mg/kg) and Xylazine (5 mg/kg) and placed inside a custom-made double-wall sound-proof room during the exposure period. Broadband white noise (1–100 kHz, 120 dB SPL re 20 μ Pa) was generated using a function generator (33500B, Agilent, USA). The noise was then amplified before it was fed into a small loudspeaker (AKG model CK 50, 1.5 cm in diameter, 1.2 g, frequency response 1–100 kHz, flat over 10–80 kHz). The noise was presented under free-field acoustic conditions and calibrated with a 1/4-inch microphone (4939, B&K, Denmark) using a measuring amplifier (2610, B&K, Denmark).

The mice were separately housed in a cage (L28 x W25 x H20 cm) during the exposure period. The noise (1–20 kHz, 110 dB SPL) was generated by the same function generator (33500B, Agilent, USA), before being amplified and sent to the speaker (JX5, HiVi, China; frequency response 0.05–20 kHz) that was suspended above the cage. The noise level at the center of the cage was measured using a 1/4-inch microphone (4939, B&K, Denmark) and a measuring amplifier (2610, B&K, Denmark). The sound level in the cage varied about 1.5 dB. The mice were continuously exposed to the noise for an hour either under gaseous anesthesia (R500, RWD, China) or in wake conditions.

Acoustic stimulation

For acoustic stimulation, continuous sine waves from a function generator (33500B, Agilent, USA) were converted into pure tone pulses or bursts (10 ms with rise-decay time of 0.5 ms, delivered at five pulses/s, hereafter identified as CF sound) using a custom-made tone burst generator that was driven by a stimulator (Master 8, AMPI, Israel). The sounds were amplified after passing through a decade attenuator (LAT45, Leader, Japan) prior to being fed into a small loudspeaker (AKG model CK 50, 1.5 cm in diameter, 1.2 g, frequency response 1–100 kHz). The loudspeaker was placed 20 cm away from the animal's ear and 30° contralateral to the recording site. The loudspeaker was calibrated using a 1/4-in. microphone (4939, B&K, Denmark) placed at the animal's ear using a measuring amplifier (2610, B&K, Denmark).

Auditory brainstem response (ABR) measurement

The ABRs were measured in a custom-made double-wall sound-proof room with a temperature range of 28–30 °C.

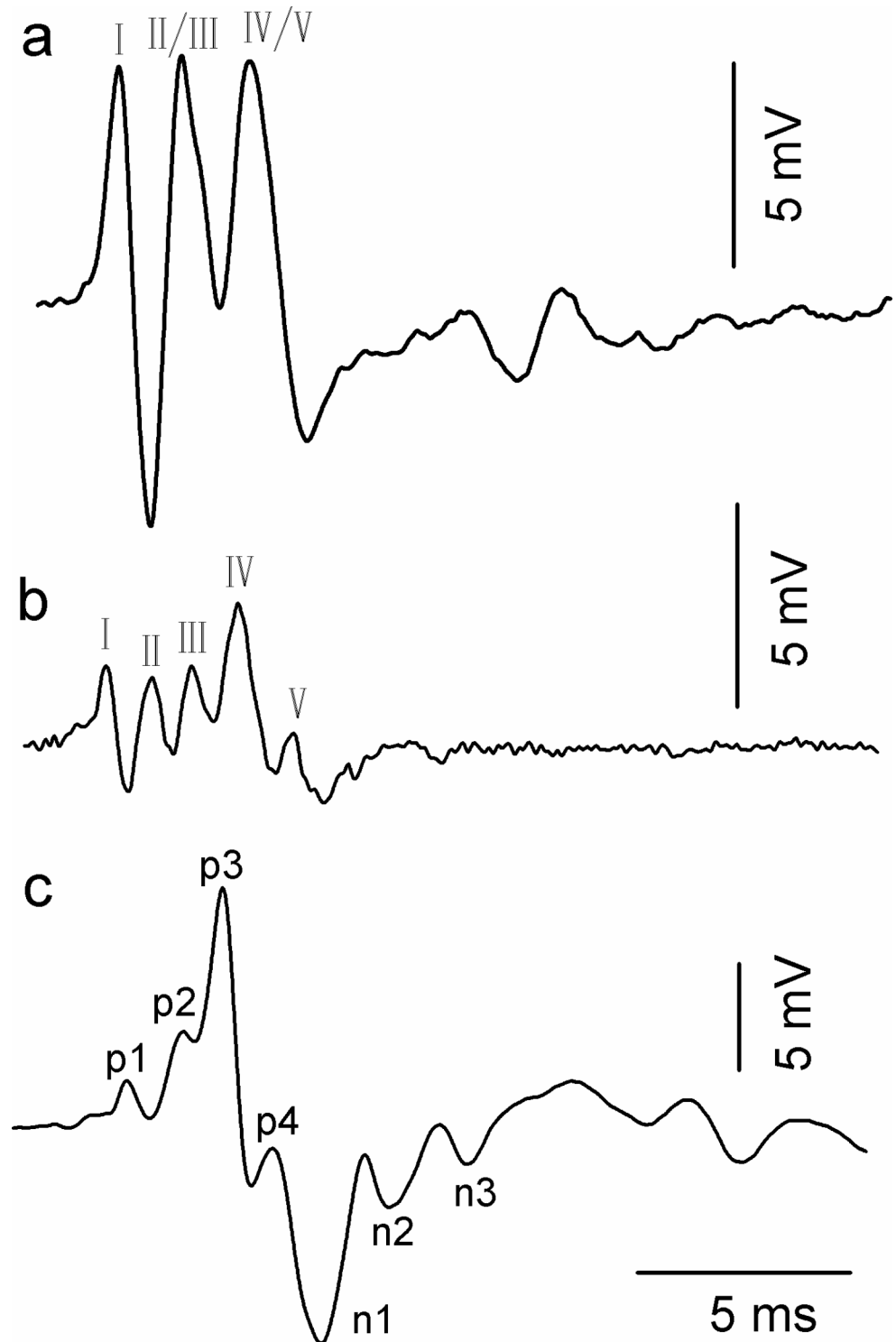
The ceiling and inside walls of the room were covered with 8-cm convoluted polyurethane foam to reduce echoes. A mixture of Nembutal (40 mg/kg) and Xylazine (5 mg/kg) was administered to the bat before it was placed inside a holder. Additional doses of the mixture were administered during later phases of recording if the bats exhibited signs of discomfort. Three needle-electrodes (NS-S83018-R9-10, Rochester, USA) were subcutaneously placed on the bat's head, positioned as follows: the recording electrode was inserted at the caudal midline of the head, close to the brainstem; the reference electrode was put at the dorsal midline of the head between the ears; the ground electrode was placed directly into the base of the right ear. As reported in our previous study (Zou et al. 2023), the ABR waveform of *H. pratti* consisted of five peaks (wave I, II, III, IV and V), whereas waves II and IV were usually fused with wave III and wave V (Fig. 1a). Since the recording electrode was positioned at the midline and not skewed to one of the ears and both ears were exposed in free-field conditions, the response was always representing the better ear (if any). For the mice, the recording electrode was inserted subcutaneously at the vertex, and the positions for reference and ground electrodes were the same as the bat. The ABR waveform of CBA/Ca mice consisted of five peaks (I, II, III, IV, and V) (Fig. 1b). At the end of recording session, the electrodes were manually removed and cleaned with 75% ethanol. No evidence of infection was noted at the sites of needle insertion.

The ABR signal was amplified (10,000x) and filtered (0.3–3.0 kHz) using a biological electrical signal amplifier (ISO-80, WPI, USA). It was digitized using an Analog-to-Digital converter that was equipped with a data acquisition system (Digidata 1440 A, Axon, USA; sampling rate, 100 kHz) before being stored in a computer database (Kaitian 4600, Lenovo, China). The signal also was monitored using an oscilloscope (DSO-X 2014 A, Agilent, USA). The PCLAMP 8.1 software (Axon Instruments, USA) was employed to replicate all stimuli 256 times at a repetition rate of 5 Hz to collect the mean ABR signals.

ABR threshold estimation

The sound frequencies that were used to obtain ABRs ranged from 5 to 80 kHz. At each sound frequency, the intensity varied from 0 dB SPL to the maximal available one. The sound frequency was kept constant while the sound intensity increased in 10-dB steps until a distinct ABR waveform was observed. The sound intensity was then reduced in 2-dB steps unless the ABR waveform was no longer visible. The lowest sound intensity which could evoke a visible ABR was deemed as the ABR threshold at the sound frequency. The ABR thresholds were established at a series of

Fig. 1 Representative ABR and AEP waveforms. (a) A representative ABR waveform from *H. pratti* stimulated with 12-kHz, 104-dB SPL, and 10-ms pure tones. (b) A representative ABR waveform from CBA/Ca mice stimulated with 12-kHz, 70-dB SPL, and 10-ms pure tones. (c) A representative AEP waveform from *H. pratti* stimulated with 12-kHz, 90-dB SPL, and 10-ms pure tones



frequencies to generate the audiogram for each bat. The frequencies started at 5 kHz and increased to 55 kHz with step sizes of 3 to 5 kHz; from 55 to 62 kHz, the step size was 0.2 to 0.5 kHz, which then reverted to 3 to 5 kHz for frequencies from 62 to 80 kHz. A small step size was used for 55 to 62 kHz because the dominant frequency was ~60 kHz, so the thresholds were expected to change drastically in this region. The threshold shifts were calculated as post-exposure thresholds minus pre-exposure thresholds.

Auditory evoked potentials (AEPs) recording

AEPs were recorded in the inferior colliculus (ICs) using a metal electrode (IRM23E01, WPI, Sarasota, USA). After being anaesthetized with a mixture of Nembutal (40 mg/kg) and Xylazine (5 mg/kg), the bat's head was fixed with a set screw, and then a small hole (200–500 μm) was made in the skull above the IC to allow for orthogonal insertion of the electrodes. Additional doses of the anesthetic mixture were intraperitoneally administered during later phases of recording whenever bats showed signs of discomfort. A local anesthetic (lidocaine) was applied to the open wound area to alleviate any pain. The recording depth was read from the scale of a microdrive (David Kopf Instrument, model 640, USA). The indifferent electrode was placed in the neck muscles. The recording system of the auditory evoked potential was comprised of the following: a biological electrical signal amplifier (ISO-80, WPI, USA); an Analogue-to-Digital converter that was equipped with a data acquisition system (Digidata 1440 A, Axon, USA); an oscilloscope (DSO-X 2014 A, Agilent, USA); and a computer (Kaitian 4600, Lenovo, China). Based on the single neuron extracellular recording from our previous studies (Cui et al. 2021; Zhang et al. 2023), this study was performed on the central nucleus of IC, and at a recording depth of 1200 μm . The AEP signal was amplified (1000x) and filtered (0.3–1.0 kHz) using the biological electrical signal amplifier prior to being averaged to 256 stimulus presentations using the PCLAMP 8.1 software (Axon Instrument, USA). For each stimulation, the AEPs were recorded twice to improve the accuracy of the recordings. The AEP responses were typically consisted of 2–4 positive peaks followed by 2–4 negative peaks at high sound intensities (Fig. 1c). The AEP threshold was estimated as the ABR threshold. The skin was stitched back to the normal position and the bat was placed into a wire mesh cage with a wire bottom (dimensions: 0.8 m \times 0.8 m \times 0.8 m) in the animal room at the end of the experiment. The bat was then fed with food and water ad libitum.

Data analysis

The data obtained through ABRs and AEPs recordings were analyzed using Clampfit 8.1. It was plotted using SigmaPlot, version 10.0 (Systat Software, San Jose, CA, USA). The results were expressed as means \pm standard error (means \pm SE). Statistical analysis of the results was done using SPSS version 13.0 (SPSS, USA). Nonparametric tests were used due to the non-normal distribution of the data. Friedman tests were used to determine the statistical significance of ABR thresholds and ABR wave I amplitudes to 90 dB SPL sound stimulation which were tested before and after the noise exposure. Mann-Whitney tests were applied to test for sexual dimorphism of ABR thresholds, and for comparing ABR thresholds and wave I amplitudes between groups of CBA mice at a single recording time. In all tests, $p < 0.05$ was considered statistically significant.

Results

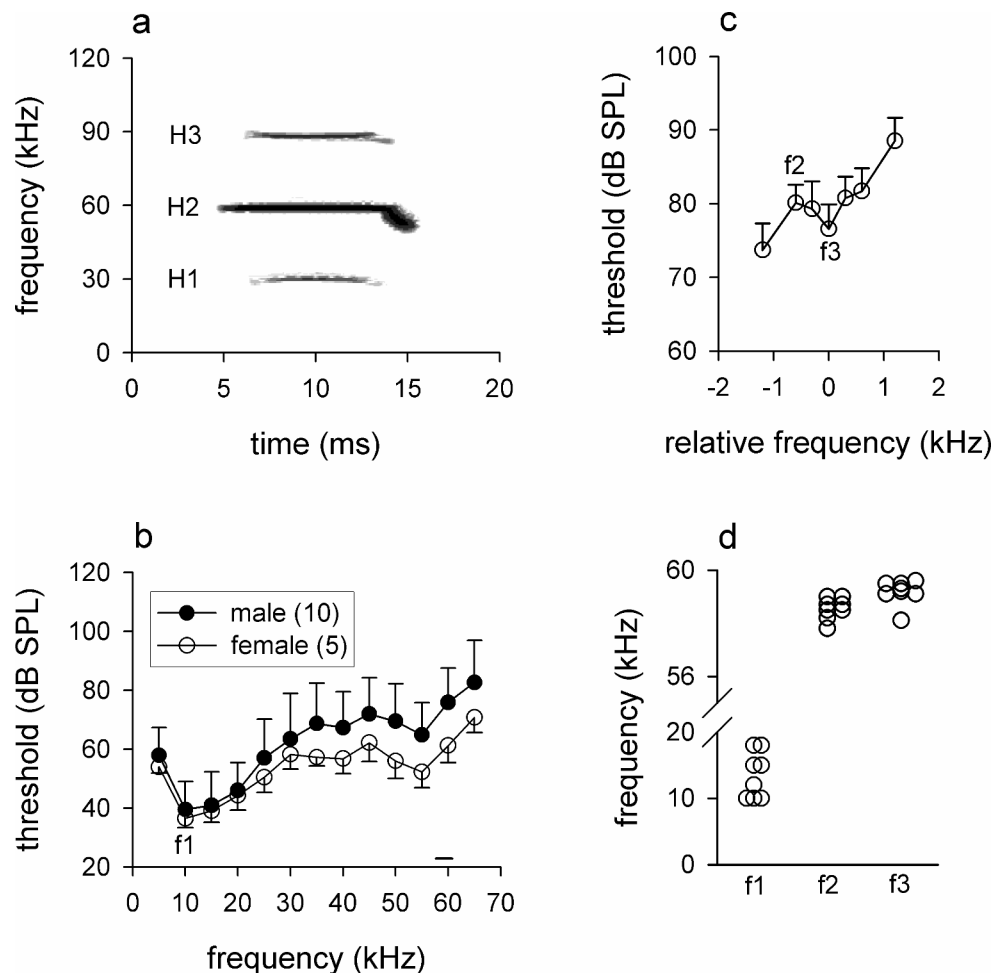
The echolocation signals of *H. pratti* typically consisted of three harmonics (H1 to H3), and each harmonic included a CF (CF1-CF3) component followed with a brief downward FM sweep (FM1-FM3) (Fig. 2a). The CF2 was 59.3 ± 0.5 (58.1–60.9) kHz for *H. pratti* used in this study.

No sexual dimorphism of ABR Audiogram in *H. pratti*

Most previous studies on auditory physiology in echolocating bats were performed on bats of both sexes, tacitly confirming the absence of sexual dimorphism in auditory signal processing. In the present study, the ABR audiograms were measured in ten male and five female *H. pratti* (Fig. 2b). The shape of the audiogram was similar for both males and females. The audiogram had two sensitive frequency areas around 10–15 kHz and 55 kHz, which were separated by a relatively insensitive area. ABR thresholds were almost the same for frequencies that ranged from 5 to 20 kHz. However, the males had slightly higher ABR thresholds for frequencies above 20 kHz. Statistical analysis showed that there was no significant difference between ABR thresholds of the males and females for all the frequencies that were tested ($p > 0.05$ for all the tested frequencies). This indicated that there was no sexual dimorphism of ABR audiogram in *H. pratti*. Thus, the experiments that followed were conducted on *H. pratti* of both sexes.

Early studies on long CF-FM bats reported a specialization in their audiogram, which had a minimal threshold near the resting frequency and a distinct maximal threshold a few kHz below (Schnitzler and Denzinger 2011). Moreover, the threshold changes between the maximal and minimal could

Fig. 2 Spectrogram and audiogram of *H. pratti*. **(a)** The spectrogram of *H. pratti*. H1, first harmonic; H2, second harmonic; H3, third harmonic. **(b)** Comparisons of audiograms of male and female *H. pratti*. The horizontal line indicates the range of the resting frequencies. **(c)** Threshold curve for stimulus-frequencies around each bat's resting frequency. **(d)** Distribution of f1, f2, and f3. f1, the most sensitive frequency in the first sensitive area; f2, the most insensitive frequency before resting frequency; f3, the resting frequency



nearly reach 40 dB for *Rhinolophus ferrumequinum* (Long and Schnitzler 1975). In this study, this specialization was checked in *H. pratti* by measuring thresholds for frequencies around the resting frequency in eight bats (four males). The sound stimulation frequencies were varied by 0.3 kHz from the resting frequency of each bat to obtain the threshold curve, and an only ~10 dB threshold increase at 0.9 kHz below the resting frequency was found (Fig. 2c).

The auditory sensitivity was unimpaired for *H. pratti* that were exposed to intense broad-band noise under anesthesia condition

Prior behavioral and electrophysiological studies showed that some species of echolocating bats can maintain their auditory sensitivity after exposure to intense noise. However, the animals were awake during noise exposure in nearly all these previous studies (Simmons et al. 2016, 2017, 2018; Hom et al. 2016; Liu et al. 2021). Echolocating bats are highly vocal animals, and the MEM starts to contract before each vocalization and relaxes after vocalization, which can attenuate sound energy across the ossicular

chain (MEM reflex) (Jen and Suga 1976). The vocalization-induced MEM reflex may be negligible in protecting the auditory system from intense noise for low-duty-cycle FM bats or other non-highly vocal animals. However, it may participate in the unimpaired auditory sensitivity when high-duty-cycle CF-FM bats are exposed to intense noise. To verify the possible function of vocalization-induced MEM reflex, *H. pratti* were anesthetized using a mixture of Nembutal and Xylazine, during exposure to noise. The vocalizations were completely inhibited during the anesthetic condition, indicating the vocalization-induced MEM reflex could not perform.

To test whether the bats suffer from permanent hearing loss, eight anesthetized *H. pratti* (four males) were exposed to 120 dB SPL broad-band white noise for an hour, and their ABR thresholds and ABR responses to 90 dB SPL pure tones were measured one day (24 h) before and two weeks after noise exposure. To minimize recording time, only the ABR thresholds and ABR responses to 90 dB SPL pure tones to three representative frequencies were measured. The representative frequencies were as follows: the most sensitive frequency in the first sensitive area (f1 for

convenience); the most insensitive frequency before resting frequency (f2 for convenience); and the resting frequency (f3 for convenience), and the distribution of f1, f2, and f3 were shown in Fig. 2d. The data showed that there were no significant shifts in ABR thresholds two weeks after noise exposure for f1 (53.4 ± 1.1 vs. 50.8 ± 1.2 dB SPL, $p > 0.05$; Fig. 3a left), f2 (81.1 ± 2.7 vs. 78.9 ± 2.4 dB SPL, $p > 0.05$; Fig. 3a middle), and f3 (71.9 ± 3.2 vs. 69.4 ± 3.5 dB SPL, $p > 0.05$; Fig. 3a right). This indicated that *H. pratti* had no permanent hearing loss when it was exposed to noise under anesthesia. Furthermore, the ABR wave I amplitude had no significant change after noise exposure for f1 (1.2 ± 0.3 vs. 1.1 ± 0.3 μ V, $p > 0.05$; Fig. 3b left), f2 (0.4 ± 0.1 vs. 0.4 ± 0.1 μ V, $p > 0.05$; Fig. 3b middle), and f3 (0.8 ± 0.2 vs. 0.7 ± 0.1

μ V, $p > 0.05$; Fig. 3b right), suggesting no hidden hearing loss.

Previous studies on marine mammals showed that recovering auditory sensitivity after noise exposure could take 10–105 min (Nachtigall et al. 2004; Mooney et al. 2009). Meanwhile, possible NIHL in Japanese house bats (*Pipistrellus abramus*) was studied at post-noise exposure intervals of 0 and 30 min (Simmons et al. 2015). To be comparable with previous studies, ABR thresholds in this study were measured before as well as 2 min, 30 min, and 60 min after noise exposure in six *H. pratti* (three males). The data showed that the noise exposure induced no significant shifts in thresholds at any of these three time points for f1 ($p > 0.05$; Fig. 3c, circle) and f3 ($p > 0.05$; Fig. 3c, square). However, the noise slightly but significantly raised

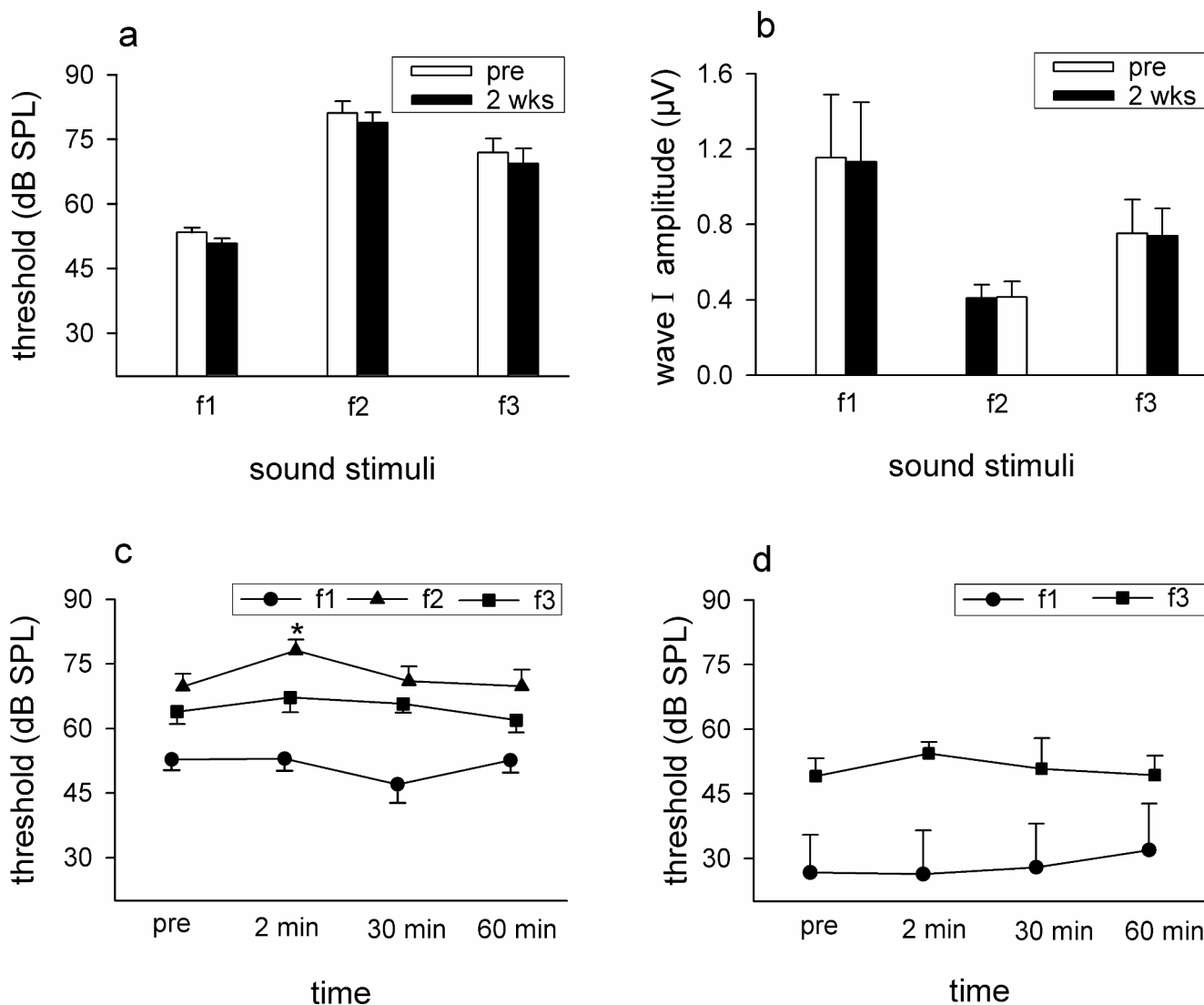


Fig. 3 Unimpaired auditory sensitivity of *H. pratti* after exposure to broad-band noise under anesthesia conditions. **(a)** ABR thresholds before and two weeks after exposure to broad-band noise. **(b)** ABR wave I amplitude before and two weeks after exposure to broad-band

noise. **(c)** ABR thresholds before and 2, 30, and 60 min after exposure to broad-band noise. **(d)** AEP thresholds before and 2, 30, and 60 min after exposure to broad-band noise. *, $p < 0.05$

the thresholds 2 min after exposure to noise when stimulated with f2 (69.7 ± 2.9 vs. 78.1 ± 2.5 dB SPL, $p < 0.05$), which recovered 30 min later (Fig. 3c triangle). When flying, CF-FM bats lower the frequency of their emitted pulses to compensate for Doppler shifts caused by their own movements. Compared to the extremely sensitive to echo frequency (near f3), the auditory systems of CF-FM bats are less sensitive to the pulse frequency (near f2), which help the bats get information from weak echoes after intense pluses (Suga 2018). Considering that f2 was the most insensitive frequency, this lack of sensitivity may help the bat to obtain information from returning echoes (Pollak et al. 1972). Therefore, it was extrapolated that the short time threshold increase would not impair the bat. As such, a conclusion was made that *H. pratti* can maintain their auditory sensitivity even a short time after exposure to noise under anesthesia.

Auditory system impairment induced by exposure to noise could also be measured by AEPs from individual auditory nuclei, and the most severe threshold shifts were in the inferior colliculus (Simmons et al. 2015; Syka and Popelar 1982). As highlighted earlier, the ABR threshold had no significant shift for f1 and f3 even 2 min after noise exposure. Therefore, further evaluations were done to determine possible noise impairment by measuring the AEP thresholds from the IC before as well as 2 min, 30 min, and 60 min after exposure to noise in four more *H. pratti* (two males). The data showed that the exposure to noise did not cause significant threshold shifts at all the three time points for both f1 ($p > 0.05$; Fig. 3d circle) and f3 ($p > 0.05$; Fig. 3d square).

Taken together, the data from this study suggested that auditory sensitivity was unimpaired for *H. pratti* exposed to broad-band intense noise under anesthesia. This indicates that the vocalization-induced MEM reflex did not take part in unimpaired auditory sensitivity.

The auditory sensitivity was more seriously impaired for CBA/Ca mice exposed to intense noise under anesthesia conditions

The data from this study suggest that auditory sensitivity was unimpaired for *H. pratti* exposed to broad-band intense noise under conditions of anesthesia. However, whether the unimpaired sensitivity was due to the anesthetization was unclear. Therefore, comparisons in auditory sensitivity after exposure to intense noise under wake and anesthesia conditions in CBA/Ca mice were made. We chose CBA/Ca mice as a comparison because this strain exhibits NIHL (Park et al. 2024) and can emit and hear ultrasounds (Sangiromo et al. 2020).

A total of 12 male CBA/Ca mice were exposed to 110 dB SPL noise (1–20 kHz, 1 h) or sham exposed (six mice each group) while they were awake. The ABR thresholds and ABR responses to 90 dB SPL pure tones were measured 24 h before as well as 24 h, one week, and two weeks after exposure. The threshold shifts were around the zero line for the sham exposure group at 24 h, one week, and two weeks after exposure (Fig. 4a–c, unfilled circle). However, the thresholds significantly shifted 24 h after exposure to noise for sound stimulation frequencies of 30, 40, and 50 kHz (Fig. 4a, filled circle). The shifts in ABR thresholds were partially recovered a week after exposure to noise (Fig. 4b, filled circle) and fully recovered two weeks after noise exposure (Fig. 4c, filled circle).

Twelve more male CBA/Ca mice were exposed to 110 dB SPL white noise or sham exposed (six mice each group) under anesthesia conditions. The ABR thresholds and ABR responses to 90 dB SPL pure tones were also measured 24 h before as well as 24 h, one week, and two weeks after exposure. The ABR threshold shifts were also around the zero line for sham exposure group at 24 h, one week, and two weeks after exposure (Fig. 4d–f, unfilled circle). The ABR thresholds significantly shifted 24 h after exposure to noise for sound stimulation frequencies of 20, 30, 40, and 50 kHz (Fig. 4d, filled circle). A week after exposure to the noise, the ABR threshold for the sound stimulation frequency of 20 kHz was recovered. Meanwhile, the ABR thresholds were still significantly shifted at sound stimulation frequencies of 30, 40, and 50 kHz (Fig. 4e, filled circle). Two weeks after exposure to noise, the ABR threshold shifts were completely recovered, except for the sound stimulation frequency of 50 kHz (Fig. 4f, filled circle).

Comparisons between ABR threshold shifts induced by exposure to noise under wake and anesthesia conditions were made (Fig. 4g–i). The ABR threshold shifts for anesthetized mice were significantly higher than for those that were awake, for sound stimulation frequencies of 40 and 50 kHz at 24 h, one week, and two weeks after exposure. The same applied for the sound stimulation frequency of 30 kHz, 24 h and one week after exposure to noise as well as 5 kHz, 24 h after exposure. These results indicated that the noise exposure under conditions of anesthesia shifted the ABR threshold more than when the mice were awake.

Further comparisons were made between the ABR wave I amplitude to 90 dB SPL pure tones at 24 h before as well as 24 h, one week and two weeks after exposure in the four groups of mice. For the mice that were exposed while they were awake, the ABR wave I amplitude to 12 kHz (Fig. 5a left) and 30 kHz (Fig. 5a right) sound stimuli had no significant change after the sham exposure. No significant change was noted in the ABR wave I amplitude to 12 kHz sound stimuli after exposure to noise (Fig. 5b left). However, the

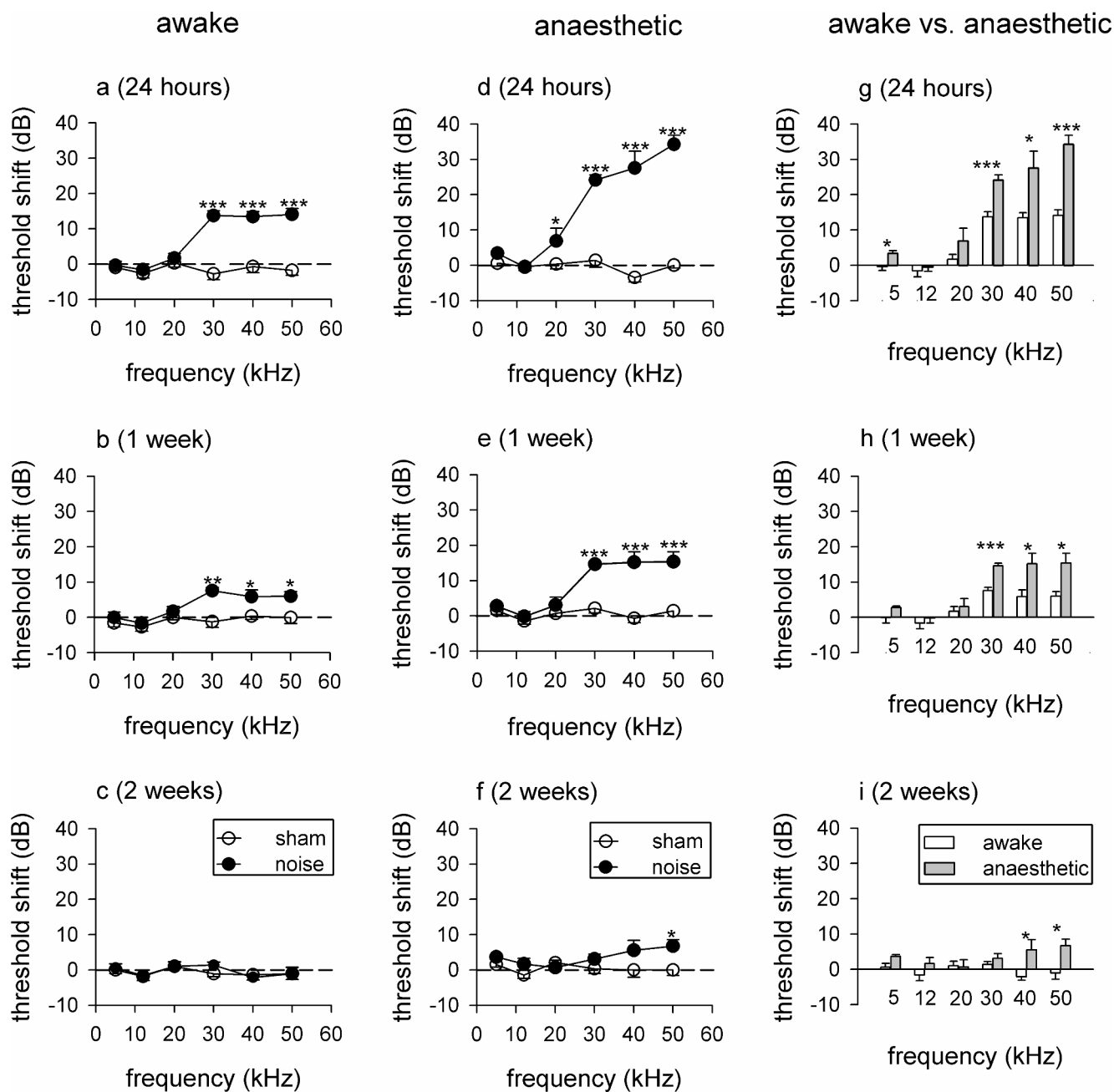


Fig. 4 The intense noise exposure under anesthesia conditions shifted the ABR threshold more than the waking state for CBA/Ca mice. **(a-c)** Comparison of ABR threshold shifts exposed (filled circle) or sham-exposed (unfilled circle) to intense noise under the wake conditions and evaluated at 24 h, one week, and two weeks. **(d-f)** Comparison of the ABR threshold shifts exposed (filled circle) or sham exposed

(unfilled circle) to intense noise under anesthesia conditions and evaluated at 24 h, one week, and two weeks. **(g-i)** Bar histograms showing the comparison of ABR threshold shifts exposed to intense noise under awake (white filled) and anesthesia (gray filled) state and evaluated at 24 h, one week, and two weeks. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

ABR wave I amplitude to 30 kHz sound stimuli significantly decreased 24 h after exposure to noise, and recovered one week and two weeks after (Fig. 5b right). For the mice exposed under conditions of anesthesia, the change in the ABR wave I amplitude was comparable to that of the mice that were exposed in the wake state (Fig. 5c and d left). However, the ABR wave I amplitude to the 30 kHz sound

stimuli did not recover even two weeks after exposure to noise (Fig. 5d right). This indicated that exposure to noise under anesthesia conditions lowered the ABR wave I amplitude more than wake conditions.

In summary, the noise exposure under anesthesia conditions would significantly reduce auditory sensitivity more than the wake state. Therefore, the unimpaired auditory

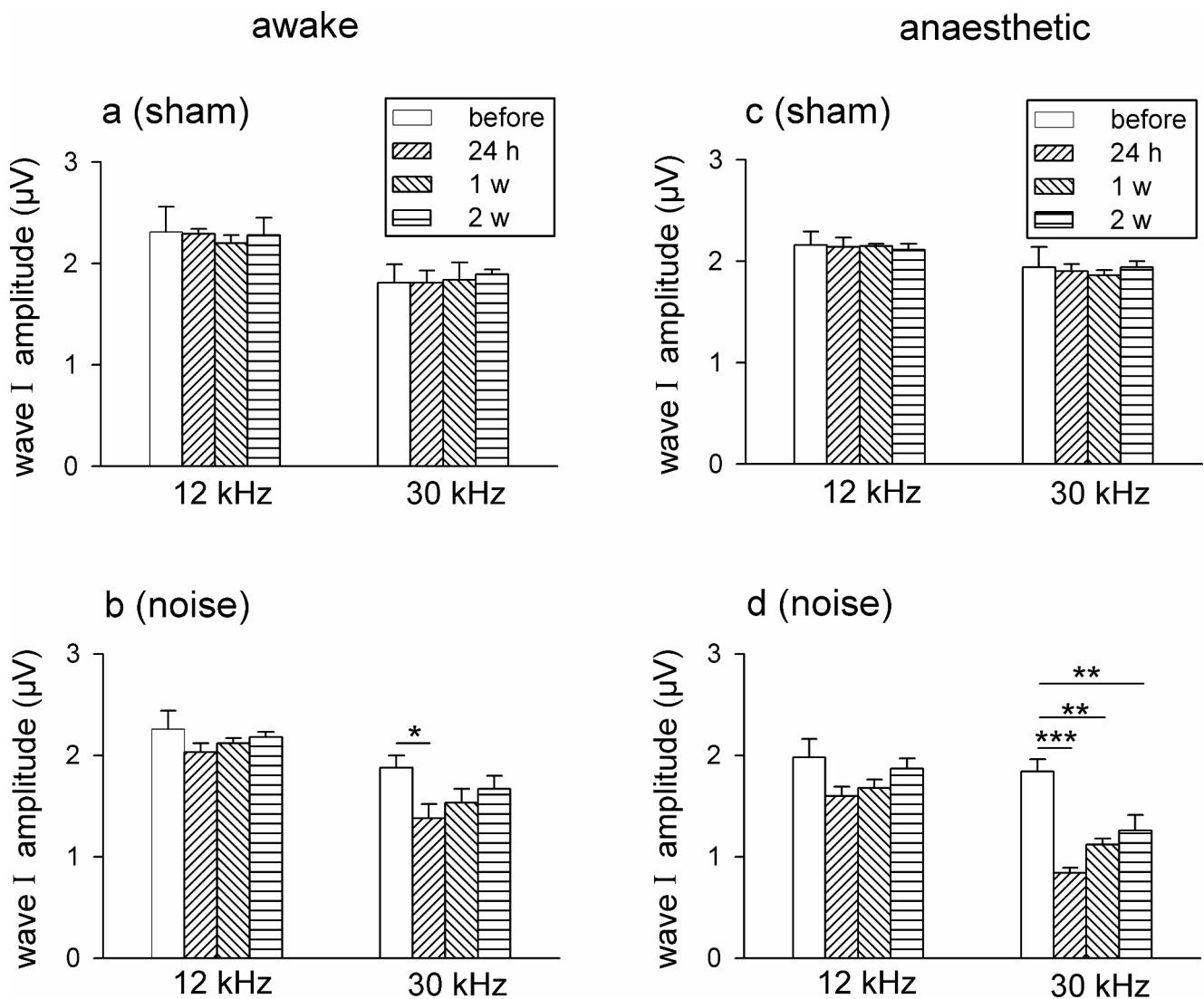


Fig. 5 The noise exposure under anesthesia conditions reduced the ABR wave I amplitude more than wake conditions for CBA/Ca mice. (a–b) Bar histograms showing the comparison of the ABR wave I amplitude sham exposed (a) and exposed (b) to intense noise under the wake conditions and evaluated at 24 h, one week, and two weeks. (c–d)

Bar histograms showing the comparison of the ABR wave I amplitude sham exposed (c) and exposed (d) to intense noise under conditions of anesthesia and evaluated at 24 h, one week, and two weeks. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$

sensitivity after exposure to intense noise under conditions of anesthesia in *H. pratti* was not due to anesthetization. Thus, vocalization-induced MEM reflex did not contribute to the unimpaired auditory sensitivity after *H. pratti* was exposed to intense noise.

***H. pratti* can maintain auditory sensitivity to non-echolocating frequencies after exposure to band-limited intense noise**

The cochlear hair cells operate as a series of bandpass auditory filters. Thus, a noise can seriously impact a hair cell when its spectrum overlaps with its passband. The more the overlap, the greater the impact. Therefore, the fact that

auditory sensitivity was unimpaired in *H. pratti* exposed to intense broad-band noise may be explained by less overlap between the noise spectrum and passband of the hair cells. To test this possibility, *H. pratti* were exposed to band-limited noise. Our previous study showed that *H. pratti* had high frequency selectivity around 60 kHz (Cui et al. 2021) due to the auditory fovea, so the passband of the hair cells tuned around 60 kHz would be narrow. In addition, low-frequency noise exposure could cause high-frequency hearing loss (Liu et al. 2022). Therefore, in this study, *H. pratti* were exposed to 1–20 kHz band-limited noise (110 dB SPL, 1 h), and their auditory sensitivity was tested at sound frequencies of 5 kHz, f1, and 30 kHz before as well as 2 min, 30 min, and 60 min after exposure. The data showed that the

ABR threshold slightly increased at 2 min after exposure for sound stimulation frequencies of 5 kHz and 30 kHz, which was consistent with behavioral studies on the big brown bats (*Eptesicus fuscus*) (Simmons et al. 2017, 2018). However, the threshold shift was only around 6 dB (Fig. 6a), which was defined as the minimum threshold shift for NIHL in marine mammals and in other mammals. No significant change was noted for the ABR wave I amplitude after exposure to noise for f1, 5 kHz, and 30 kHz sounds at all time-points that were tested (Fig. 6b). These data suggested that the auditory fovea did not contribute to the unimpaired auditory sensitivity after *H. pratti* was exposed to intense noise.

Discussion

The data from this study showed that there was no sexual dimorphism in the bat's ABR audiogram. Therefore, noise exposure experiments with *H. pratti* were performed on both sexes. After exposure to broad-band intense noise under anesthesia conditions, *H. pratti* could maintain their auditory sensitivities, as indicated electrophysiologically, two weeks and even two min after the exposure. While the CBA/Ca mice had more seriously impaired auditory sensitivity when exposed to intense noise under conditions of anesthesia than the wake state, the data from this study suggest that the unimpaired auditory sensitivity in *H. pratti* after broad-band noise exposure was not due to the anesthesia. Considering that the vocalization-induced MEM reflex could not work under anesthesia conditions, we concluded that the vocalization-induced MEM reflex did not contribute to the unimpaired auditory sensitivity in *H. pratti* after exposure to intense noise. *H. pratti* were further exposed to band-limited noise, whose spectrum did not overlap with

that of the bat's echolocation calls. Moreover, *H. pratti* could also maintain their auditory sensitivity to sound stimulation of frequencies in the noise spectrum and one-half octave higher than the spectrum. This suggests that the auditory fovea also did not contribute to the unimpaired auditory sensitivity after exposure to intense noise.

Sexual dimorphism in auditory sensitivity

Sexual dimorphism in auditory sensitivity has been studied in a few species, including mice (Park et al. 2024), rats (Charlton et al. 2019), frogs (Liu et al. 2014), birds (Yeh et al. 2023), and bats (Lattenkamp et al. 2021). While CBA mice (Park et al. 2024) and estrildid songbirds (Yeh et al. 2023) showed no sexual dimorphisms in their audiograms, the male Long-Evans rats exhibited lower auditory sensitivity than females (Charlton et al. 2019). There was no sexual difference in the audiograms of frogs of most species, excepting the concave-eared torrent frog, *Odorrana tormota*, and the bullfrog, *Rana catesbeiana*, which have sexually dimorphic middle ears (Mason et al. 2003; Shen et al. 2011). The audiogram of the large odorous frog, *Odorrana graminea*, was also markedly different between males and females. However, whether they also differ in their middle ears was unknown (Liu et al. 2014). Lattenkamp et al. (2021) showed that the ABR thresholds in bats were more sensitive for females than males in a frequency range corresponding to pup isolation calls in *Saccopteryx bilineata* and *Desmodus rotundus*, while there were no sexual dimorphisms in *Glossophaga soricina* and *Thyroptera tricolor*, which was in accord with our findings in *H. pratti*. Sexual dimorphism in the acoustic signals was reported in many bat species (Chaverri et al. 2018), and the auditory behavior thresholds of female *Phyllostomus hastatus* were lower than

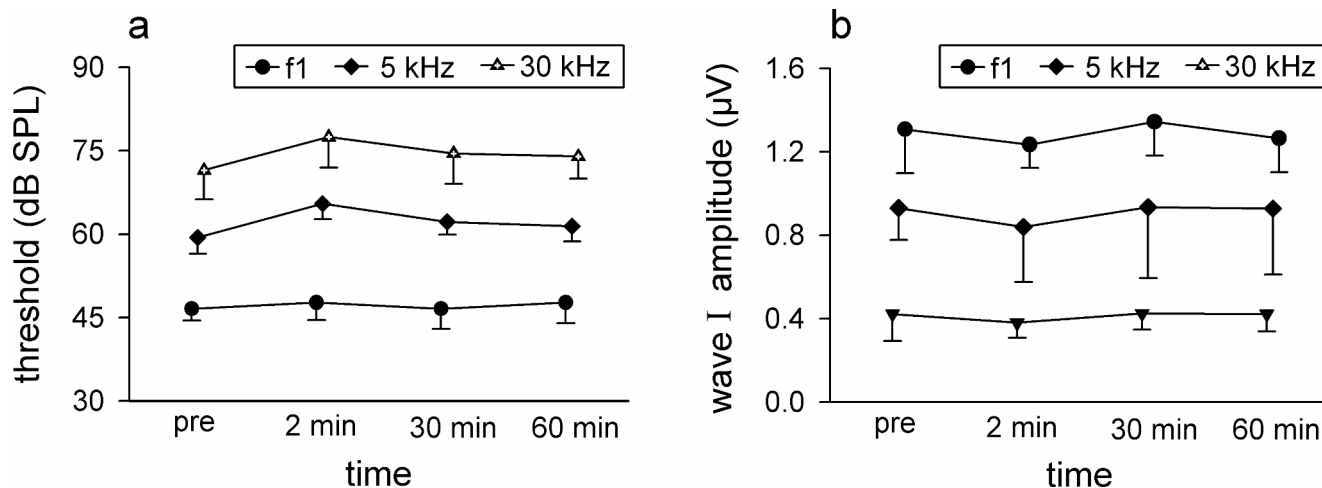


Fig. 6 Unimpaired auditory sensitivity of *H. pratti* after exposure to band-limited noise under conditions of anesthesia. **(a)** ABR thresholds before as well as 2, 30, and 60 min after exposure to band-limited

noise. **(b)** ABR wave I amplitude before as well as 2, 30, and 60 min after exposure to band-limited noise

the male to the frequency range of pup isolation calls (Bohn et al. 2004). Therefore, it might be reasonable that sexual dimorphism in audiograms existed in the frequency range of their social vocalizations, especially in the range of pup isolation calls. A recent study reported that Egyptian fruit bats *Rousettus aegyptiacus* exhibited some age-related hearing loss (Tarnovsky et al. 2023), indicating that age should be held comparable between male and female bats when studying sexual dimorphism in audiograms. Because the sample sizes for each sex was limited and the exact ages of bats were unknown in most of previous and present studies, we cannot conclude whether sexual dimorphism in the ABR audiogram exists in bats.

The MEM reflex

The MEM reflex is an important feedback system to auditory periphery (Mukerji et al. 2010). The MEM reflex could be evoked by passively hearing loud sounds and self-generated noise, such as vocalization or swallowing (Borg and Counter 1989). The vocalization-induced MEM reflex may protect the animal from acoustic trauma. For example, the vocalization-induced MEM reflex was found to attenuate cochlear microphonic potentials by an amount equivalent to sound pressure reductions of 20–25 dB in the CF-FM bat *Pteronotus parnellii* (Pollak and Henson 1973). However, this protective role was found to act as high pass filters that only attenuated the energy in frequencies below 57 kHz (Pollak and Henson 1973). Therefore, the MEM reflex might not be involved in the protection from high-frequency noise exposure (> 60 kHz) in *H. pratti* even under the wake conditions.

Possible mechanisms for unimpaired auditory sensitivity after intense noise exposure in echolocating bats

Both electrophysiological and behavioral results showed that echolocating bats, including FM bats (*Pipistrellus abramus* in Simmons et al. 2015; *Eptesicus fuscus* in Simmons et al. 2016; Hom et al. 2016; Simmons et al. 2017; and Simmons et al. 2018; *Myotis ricketti*, *Miniopterus fuliginosus*, and *Ia io* in Liu et al. 2021) and CF-FM bats (*Hipposideros armiger* and *Rhinolophus sinicus* in Liu et al. 2021), can maintain their auditory sensitivity after they have been exposed to intense noise. Although increasing evidence continuously confirms the high resistance to noise damage in echolocating bats, information on the underlying mechanisms remains limited.

Echolocating bats might have developed immunity to intense noise through evolutionary pressures. Intense noise would impair auditory sensitivity, which was evaluated by

an auditory threshold shift. However, the susceptibility to noise greatly varies among different species. While some species that live in quiet environments have high susceptibility to noise (for example, chinchillas, Jiang et al. 2023; rats, Li et al. 2021; mice, Wu et al. 2020), the echolocating bats (Simmons et al. 2015, 2016, 2017, 2018; Hom et al. 2016; Liu et al. 2021), which lived in continuously intense acoustic environments, were relatively resistant to noise exposure. Therefore, echolocating bats are believed to have developed immunity to intense noise by evolutionary pressures (Simmons et al. 2016). Furthermore, Tarnovsky et al. (2023) showed that the Egyptian fruit bats exhibited age-related hearing loss, and the deterioration rates were lower for frequencies in the spectrum of their conspecific vocalizations. This further supports the assumption that bats developed immunity to intense noise via evolutionary pressures. However, the noise exposure intensity that was used in previous studies never exceeded the levels of the environmental noise of bats. Whether ultra-high intensity noise could impair the auditory sensitivity of echolocating bats may further verify the assumption concerning the role of evolutionary pressures in enhancing bats' resistance to noise.

Although our results ruled out the contribution of vocalization-induced MEM reflex to the unimpaired auditory sensitivity in *H. pratti* after exposure to intense noise, the MEM reflex induced by passively hearing loud sounds might contribute to hearing preservation in bats. Intense acoustic stimuli would contract the MEM and increase middle ear impedance, and then attenuate the intensity of sound energy reaching the cochlea (Mukerji et al. 2010). Therefore, it is possible that the echolocating bats had a strong MEM reflex to intense noise and future experiments are needed to verify it.

Overexpression of *ISLI* in cochlear hair cells might protect echolocating bats from the negative effects of intense noise. A recent study by Liu et al. (2021) compared cochlear transcriptomic data from fruit bats, mice, and echolocating bats and found that *ISLI*, which is responsible for protecting cochlear hair cells, was overexpressed in echolocating bats. The researchers further transfected the organs of Corti explants with the recombinant adenovirus expressing *ISLI* and found that *ISLI* expression could partially rescue the explants from ototoxic drugs. These results indicated that the cochlear hair cells of echolocating bats may have protective effects against intense noise. However, the hair cells of the mustached bat's cochlea could be damaged by ototoxic drugs (Kössl and Vater 2000). Further studies where echolocating bats and other species are injected with comparable doses of the ototoxic drug may be needed to verify whether the cochleas of echolocating bats are special for dealing with noise and ototoxic challenges.

The medial olivocochlear (MOC) system might participate in maintaining auditory sensitivity after echolocating bats are exposed to intense noise. The MOC efferent neurons project from the superior olivary complex to the cochlea to innervate outer hair cells, forming a negative feedback, gain-control system for cochlear amplification of sounds (Lauer et al. 2022). Enhancing the MOC system could prevent adult mice from hidden hearing loss (Boero et al. 2018) and aged mice from presbycusis (Boero et al. 2020). Furthermore, the strength of the MOC system could be handy in predicting vulnerability to acoustic injury in guinea pigs (Maison and Liberman 2000). Therefore, the MOC system of echolocating bats might play a significant role in maintaining auditory sensitivity after exposure to intense noise.

The auditory cortex (AC) has a protective effect on auditory function. The AC modulates subcortical auditory structures through the corticofugal system, which could reduce or filter cochlear responses (Elgueda and Delano 2020). Electric stimulation of cortical neurons could modulate cochlear hair cells via the corticofugal system in the mustached bat, *Pteronotus parnellii* (Xiao and Suga 2002). A recent study showed that multisession direct current stimulation of the AC could delay the progression of presbycusis in Wistar rats (Fernández Del Campo et al. 2024). Accordingly, the AC might also protect the auditory function from exposure to intense noise.

Limitations

In the present study, functioning of the vocalization-induced MEM reflex was blocked by anesthesia using a mixture of Nembutal and Xylazine, which could also affect the function of the nervous system. However, this did not skew our conclusion that the vocalization-induced MEM reflex did not take part in the unimpaired auditory sensitivity because the bats maintained their auditory sensitivity after they had been exposed to intense noise under anesthesia conditions. In addition, we did not examine the status of the cochleae of the bats, so we were not sure whether they were damaged after continuous intense noise exposure under conditions of anesthesia. Our data showed that the CBA/Ca mice had more seriously impaired auditory sensitivity when exposed to intense noise under anesthesia conditions than the wake state, we concluded that the noise exposure under anesthesia conditions would significantly reduce auditory sensitivity more than the wake state. However, the awake mice might also use some unrevealed behavioral strategies to avoid the noise, which required further studies to uncover. Furthermore, the noise susceptibility was studied in *H. prattii* and CBA/Ca mice, but experimental methods were not the same for bats and mice, including anesthetic, noise exposure, recovery time after noise exposure, and experimental

grouping, which might be the main limitation of present study.

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Author contributions Ziyang Fu contributed to the conception and design of the study, acquisition, analysis and interpretation of the data, and drafting the manuscript. Zhongdan Cui, Jianwen Zou, Yuting Zhou, Yuntu Cao, Haonan Song, Haoyue Xu, Jing Wu, and Baoling Jin contributed to acquisition, analysis, and interpretation of the data, and revising the draft critically for important intellectual content. Lijian Yang, Ya Jia, and Qicai Chen contributed to revising the draft critically for important intellectual content. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the study. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Declarations

Competing interests The authors declare no competing interests.

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