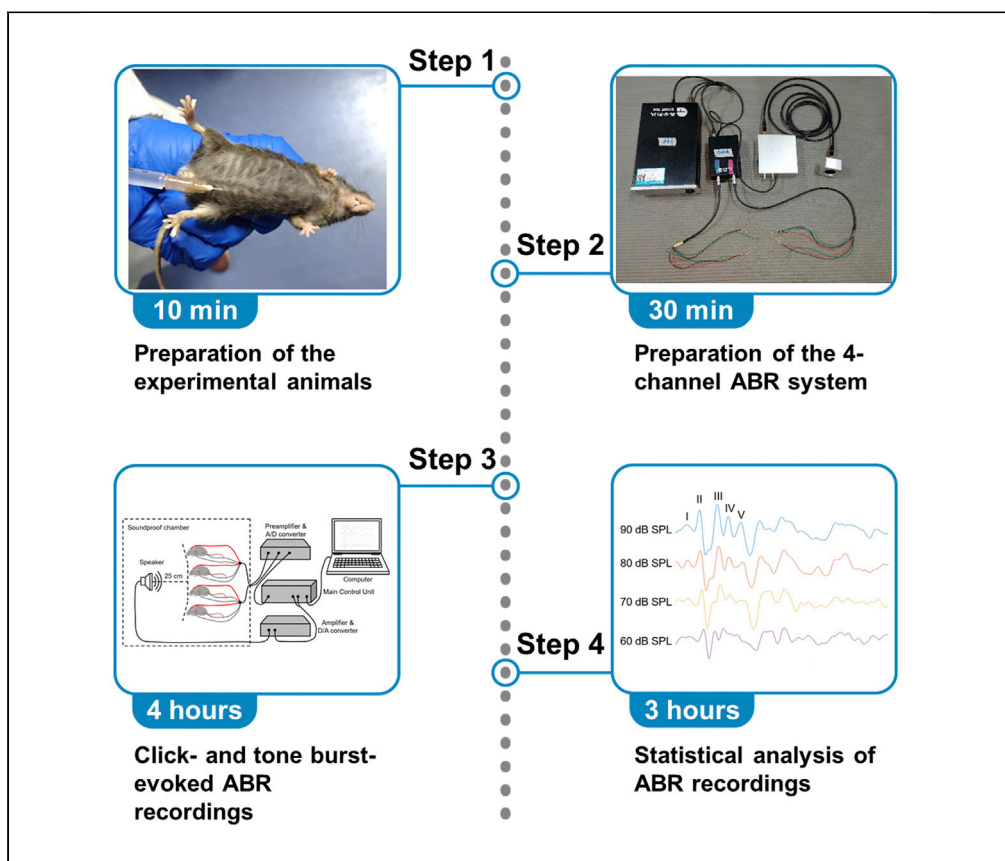


Protocol

Protocol for assessing auditory brainstem response in mice using a four-channel recording system



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Highlights

A detailed protocol for assessing the auditory brainstem response in mice

Determining threshold of click-evoked ABR

Obtaining thresholds of tone burst-evoked ABR

Identifying hearing impairment in hypothermic and middle-aged mice

Evaluating auditory sensitivity is critical to hearing research, particularly to that focusing on hearing impairment. Auditory brainstem response recording is frequently used in mice to assess auditory sensitivity and is an approach superior to the traditional techniques. Here, we describe a protocol for recording ABR in mice using four-channel equipment. We detail the procedures of animal preparation, the setup of the ABR recording system, the click- and tone burst-evoked ABR recordings, and data analysis.

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Protocol

Protocol for assessing auditory brainstem response in mice using a four-channel recording system

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SUMMARY

Evaluating auditory sensitivity is critical to hearing research, particularly to that focusing on hearing impairment. Auditory brainstem response recording is frequently used in mice to assess auditory sensitivity and is an approach superior to the traditional techniques. Here, we describe a protocol for recording ABR in mice using four-channel equipment. We detail the procedures of animal preparation, the setup of the ABR recording system, the click- and tone burst-evoked ABR recordings, and data analysis.

BEFORE YOU BEGIN

The auditory brainstem response (ABR) method has over the years been widely used in clinical and research settings to assess auditory sensitivity. It is a method that is superior to the traditional techniques, such as observing the Preyer reflex. The Preyer reflex is a simple technique involving an administration of a sound stimulus, e.g., a loud hand clap, to an experimental animal, followed by observation of the reflective reaction (Jero et al., 2001). ABR recording is recognized as an easy-to-execute, low-cost and minimally invasive technology that reliably estimates the sensitivity of hearing. The characteristic waves of ABR are labeled with Roman numerals I-V, which originate from the cochlea and/or auditory nerve, the cochlear nuclei, and other brain structures. Here, we detail the procedure for assessing hearing threshold using a four-channel ABR system in mice. As an example, we collected data from mice with normal and impaired hearing. Animal procedures were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology.

Preparation of the experimental animals

⌚ Timing: 10 min

1. Experimental animals. We use male C57 BL/6 mice in this study. The ages of the animals are specified in the figure legends. The animals are housed in a temperature-controlled room under a 12 h light/dark cycle. They have free access to rodent chow and tap water.

Note: It is not appropriate to perform ABR testing on mice that have signs of external ear canal or middle ear obstruction, or middle ear infection.



- Anesthesia. Anesthetize the mouse 2–5 min prior to ABR recording via intraperitoneal injection of Avertin (tribromoethanol, 300 mg/kg).

Note: Wear a laboratory coat, protective gloves and face mask when working with mice.

Note: Genetic background, age and body weight (e.g., obesity) of the mice may have effects on the efficacy of anesthetics (Naveilhan et al., 2001; Schuetze et al., 2019). We used male C57 BL/6 mice in the following procedures. An extra dose of Avertin (1/3 of the original dose) will be needed if the procedures last for 45 min or longer.

Alternatives: A combination of ketamine (100 mg/kg) and xylazine (10 mg/kg) is also recommended. Narcotic drugs and dosage regimens can be referred to the institutional guidelines.

- Make sure the animals are deeply anesthetized (e.g., lack of response after a tail pinch or toe pinch). Maintain this level of anesthesia.
- The animals should be placed in a soundproof chamber. Maintain the animal's body temperature at 36.5°C–38.0°C with a heating pad.

△ **CRITICAL:** Mice are prone to hypothermia due to the high ratio of body surface to body volume. When mice are deeply anesthetized, their metabolic rate will drop, which will impact the function of the inner ear and, ultimately leading to the elevation of the auditory threshold (Shaw, 1986; Williston and Jewett, 1982). Similarly, the mice should not be overheated.

Preparation of the recording equipment

⌚ **Timing:** 30 min

- The Giant multi-channel ABR system (Figure 1. Cat #GAT-ABR-003, Shenzhen Giant Technologies Company, Shenzhen, China) is used to assess 4 animals simultaneously. This device is powered by the rechargeable battery packs (see also troubleshooting).

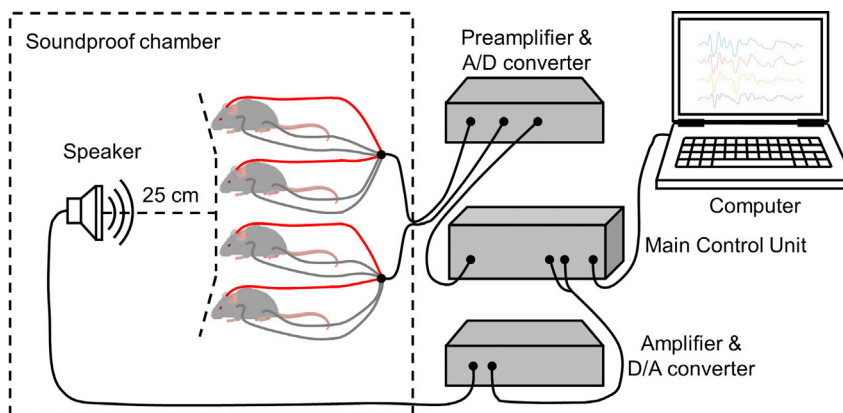


Figure 1. A schematic for auditory brainstem response setup

Four mice are anesthetized, inserted with electrodes, and placed at a distance of 25 cm between their heads and the leading edge of the loudspeaker. The soundproof chamber is illustrated as dotted line. ABR stimuli, including clicks and tone bursts, are generated by speaker amplifier and the digital/analog (D/A) converter controlled by the Giant ABR software. ABR signals are amplified, bandpass filtered, and converted using an analog/digital (A/D) converter over 512 stimuli.

Note: Make sure that the battery packs are fully charged, which usually takes ≥ 2 h, prior to use.

- Before mice are assessed, the system needs to be calibrated according to the manufacturer's [instructions](#). In doing so, data obtained from different systems or laboratories can be compared ([Burkard, 2006](#)). Place the microphone (PCB 377C01) with preamplifier (PCB 426B03) 25 cm in front of the loudspeaker.
 - Open the calibration interface of the Giant ABR software.
 - Select click or burst mode and load the corresponding configuration file. Click start to initiate calibration.
- Place the loudspeaker 25 cm in front of the mouse head in a custom-made soundproof chamber (1.2 m L x 0.8 m W x 0.9 m H). The position of the loudspeaker should be oriented perpendicular to the mouse's interaural axis.

Alternatives: Mark the position of the microphone used for calibration and place the mouse's head in the marked position.

- Scrub the ABR needle electrodes with 75% ethanol solution. Place the active electrode into the scalp subdermally at the vertex of the skull and between the ears ([Figure 2A](#)).
- The reference electrode and the ground electrode should be placed subdermally underneath the ipsilateral ear (i.e., the ear of which the ABR is assessed) and at the hindlimb ([Figures 2B and 2C](#)), respectively.

Alternatives: Place the ground electrode at the contralateral ear ([Chien et al., 2016](#)). In addition, the ground electrode can be inserted over the bregma, and the recording electrode is positioned lateral from the bregma just behind the pinna ([Jansen et al., 2013](#)). Alternatively, place the active electrode subcutaneously at the vertex, the reference electrode ventrolateral to the left ear, and the ground electrode above the tail ([Crispino et al., 2011](#)).

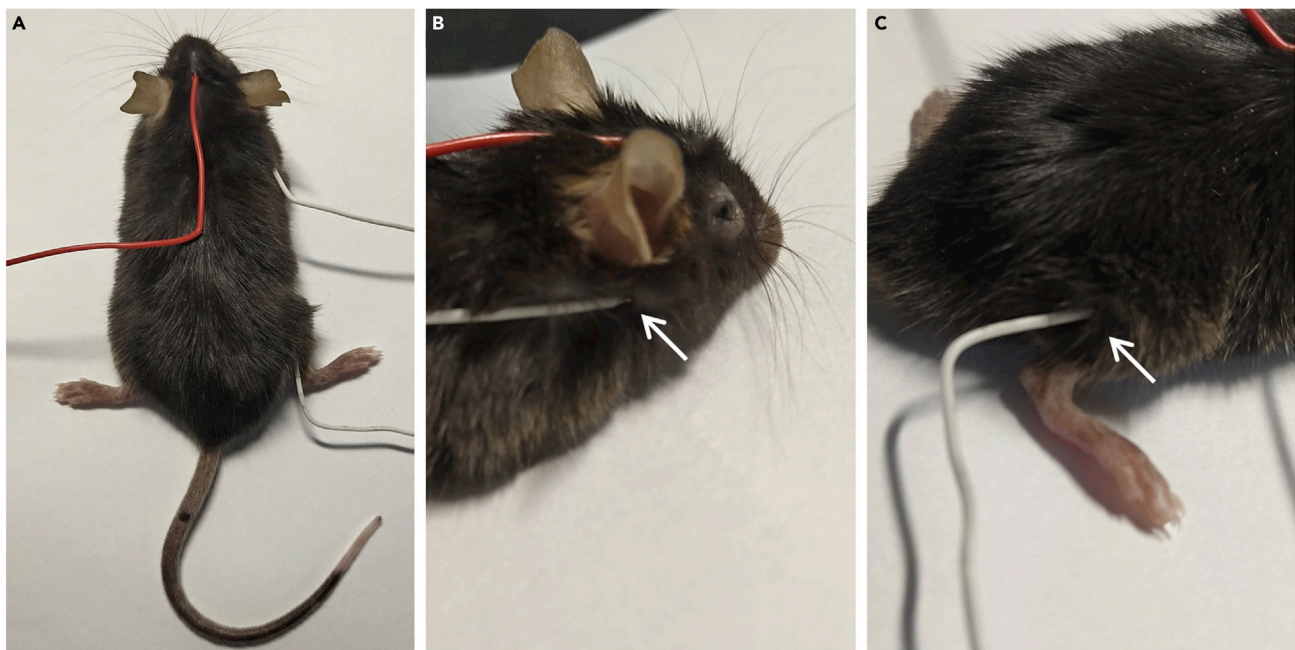


Figure 2. Positioning of the needle electrodes for ABR recording

(A) The active electrode is placed into the scalp subdermally at the vertex of the skull and between the ears.

(B and C) The positions to place reference electrode (B) and ground electrode (C) are indicated with white arrows.

10. Examine the impedance before the recording of the auditory brainstem response. If the impedance is greater than 5 k Ω , adjust the needle electrode (e.g., the depth of insertion) so that it is placed at the proper position.
11. Set the program provided with the ABR system to record tone bursts (rate of sound presentation: 14/s) at 10-dB steps from 90 dB SPL (sound pressure level) to 10 dB SPL, which is below threshold for most mice with normal hearing ability.

Note: The 10-dB down step was set based on existing empirical evidence (Jansen et al., 2013; Papesh and Hurley, 2016; Pienkowski, 2018), but can be adjusted as necessary. For example, if 10 dB is above the threshold for some of the mice, a lower intensity step can be added. Conversely, low-intensity steps may be omitted in mice with known hearing loss.

△ CRITICAL: No electronics using A.C. should be utilized in the soundproof chamber when recording ABR.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Avertin	Sigma-Aldrich	Cat #T48402
tert-Amyl alcohol	Sigma-Aldrich	Cat #152463
1x PBS	Sangon Biotech	Cat #B540626
Experimental models: Organisms/strains		
Mouse: C57BL/6	GemPharmatech	Cat #N000013
Software and algorithms		
ABR recording software	Shenzhen Giant Technologies	https://gianttek.cn/en/%e6%9c%aa%e5%88%86%e7%b1%bb-en/abr/
Data analysis software	Shenzhen Giant Technologies	https://gianttek.cn/en/%e6%9c%aa%e5%88%86%e7%b1%bb-en/abr/
SPSS software (Ver 17.0)	IBM	https://www.ibm.com/analytics/spss-statistics-software
Other		
ABR instrument	Shenzhen Giant Technologies	Cat #GAT-ABR-003
Microphone	PCB Piezotronics	Cat #377C01
Preamplifier	PCB Piezotronics	Cat #426B03
Digital thermometer for small rodent	Zhongjiao Jianyi Technology Development	Cat #TH-212
Rodent chow	HFK Bioscience	Cat #1025
T/Pump	C2Dx	Cat #TP700
Heating Pad	C2Dx	Cat #8002-062-026
Syringe filter (0.22 μ m)	Jet Bio-Filtration	Cat #FPV-203-030
1-mL syringe with 23–25 G needle	Purchased from any pharmacy	N/A
Artificial tear	Thera Tears	N/A
1% Povidone-iodine antiseptic-microbicide solution	Med Vet International	Cat #NC1802798
75% Ethanol	Purchased from any pharmacy	N/A

MATERIALS AND EQUIPMENT

Avertin stock solution		
Reagent	Final concentration	Amount
Avertin (2-2-2 tribromoethanol)	1.6 g/mL	25 g
tert-Amyl alcohol	N/A	15.5 mL
Total	N/A	15.5 mL

- Dissolve 25 g of Avertin in 15.5 mL of *tert*-Amyl alcohol.
- Seal the amber bottle to protect the solution from light. Place it on the magnetic stirrer. Stir the solution with a magnetic bead overnight (or 8–16 h) at room temperature (18°C–25°C).
- Store the solution at room temperature.

△ **CRITICAL:** Discard the stock solution if it turns yellow. This solution is stable at room temperature for about 1 yr.

Avertin working solution		
Reagent	Final concentration	Amount
Avertin stock solution (1.6 g/mL)	20 mg/mL	0.5 mL
1 × PBS	N/A	39.5 mL
Total	N/A	40.0 mL

- Add 0.5 mL of Avertin stock solution to 39.5 mL of 1 × PBS.
- Protect the solution from light. Stir the solution with a magnetic bead overnight (or 8–16 h) at room temperature.
- Sterilize the solution by passing through a 0.22- μ m filter (Jet Bio-Filtration, Guangzhou, China). Aliquot and store at 4°C in the dark. This solution may be used for up to 3 months.

Alternatives: The ABR acquisition system can also be obtained from Tucker-Davis Technologies or Intelligent Hearing Systems.

STEP-BY-STEP METHOD DETAILS

⌚ **Timing:** 4 h

This section describes the procedures to perform ABR testing using click stimuli and stimuli with varied frequency and SPL. Personal protection equipment should be worn throughout the procedures.

1. Turn on the computer and open the Giant ABR recording software (provided by the manufacturer).
2. Anesthetize 4 mice by using intraperitoneal injection of Avertin at the dose of 300 mg/kg body weight. Make sure the mice are deeply anesthetized (e.g., lack of response to toe pinch).

Note: Wear a laboratory coat, protective gloves and face mask when working with mice.

3. Place the animals on a non-electric heating pad in a soundproof chamber (see also [troubleshooting](#)), maintain the animals' body temperature at 36.5°C–38.0°C (see also [troubleshooting](#)).

△ **CRITICAL:** Throughout the experiment, check the rectal thermometer from time to time to make sure the body temperatures are in the normal range.

△ **CRITICAL:** To prevent the drying of the cornea, add a drop of artificial tear to each eye.

4. Place the ABR electrodes to the vertex (active), the ipsilateral ear (reference), and the ipsilateral hindlimb (ground) ([Figure 2](#)).

△ **CRITICAL:** Make sure that the electrode wires are tension-free. It is ideal that the needle electrode impedance should be less than 5 k Ω , and that the impedances of the electrodes are comparable.

5. Turn on the ABR recording system. Close the soundproof chamber (see also [troubleshooting](#)).
6. Use the Shenzhen Giant software provided with the ABR system to coordinate loudspeaker control as well as ABR acquisition, processing, averaging and data management. Sound signals are generated by a generator unit controlled by the software. Open the acquisition program and start data collection.
7. Start the click stimulation procedure. Recording auditory brainstem responses to 512 click stimuli. Standardize the click stimulus at 100 μ sec duration, 20/s, with a decreasing level from 90 dB SPL to 10 dB SPL in 10-dB SPL steps.
8. Once the click recording is completed, start the tone burst (20 ms duration with 1 ms rise and fall time, as well as 47 ms interval) stimulation protocol within the range of interest (e.g., 4–48 kHz).
 - a. Identify auditory thresholds for each stimulus by decreasing the SPL in 10-dB steps.
 - b. Continue recording ABR until waves I, II, III and IV cannot be discerned.
 - c. Adjust the sound pressure level up and down in 5-dB steps to identify the lowest level.
 - d. Record the response to each stimulus and obtain the mean response value over 512 measurements.

Note: If it takes more than 45 min to carry out the ABR, or, after testing the first ear, the other ear needs to be assessed, temporarily suspend the test and inject an extra amount of Avertin (1/3 of the initial dose) to maintain a proper level of anesthesia in mice (see also [troubleshooting](#)).

9. Once the recording is completed, carefully remove the needle electrodes from the mice. Place the animals in a warm cage until they are fully recovered.
10. When all tests are finished, soak the needle electrodes in a povidone iodine solution for 1 h. The electrodes were then rinsed with 75% ethanol, and kept in a sterile container.

△ CRITICAL: No electronics using A.C. should be utilized in the soundproof chamber when recording ABR.

EXPECTED OUTCOMES

The ABR recordings should be analyzed offline. The Shenzhen Giant Technologies software (integrated with the ABR system, Shenzhen Giant Technologies) is used to process the original data and to generate the ABR waveforms. The following parameters are determined: amplitude of waves, latency, inter-peak latency, and threshold. Peak latencies are defined as the time elapsed from time zero to wave peaks. Individual wave amplitude is defined as the difference between the peak and the following trough. The ABR threshold is identified as the lowest waveform where any recognizable feature can be discerned (see also [troubleshooting](#)). The hearing ability of mice ranges from 0.5 to 120 kHz; however, they are more sensitive to the frequencies between 12 kHz and 24 kHz ([Zheng et al., 1999](#)). In [Figure 3A](#), click-evoked ABR is shown. The thresholds of 8 kHz tone bursts-elicited ABR are significantly elevated in mice under hypothermia ([Figures 3B and 3C](#)). Hearing sensitivity declines progressively with aging. In literature, C57 BL/6 mice showed significant hearing impairment after 6 months of age ([Li and Borg, 1991](#)). In agreement with this observation, both the thresholds of click- and tone burst-evoked ABR are elevated in mice with hearing impairment as compared to the normal control ([Figures 4 and 5](#)).

QUANTIFICATION AND STATISTICAL ANALYSIS

⌚ Timing: 3 h

We used SPSS statistics software (Ver. 17.0, IBM, Armonk, NY) for data analysis. To examine the effect of age on ABR thresholds, the two-way analysis of variance (ANOVA) with Bonferroni's *post hoc* test was utilized.

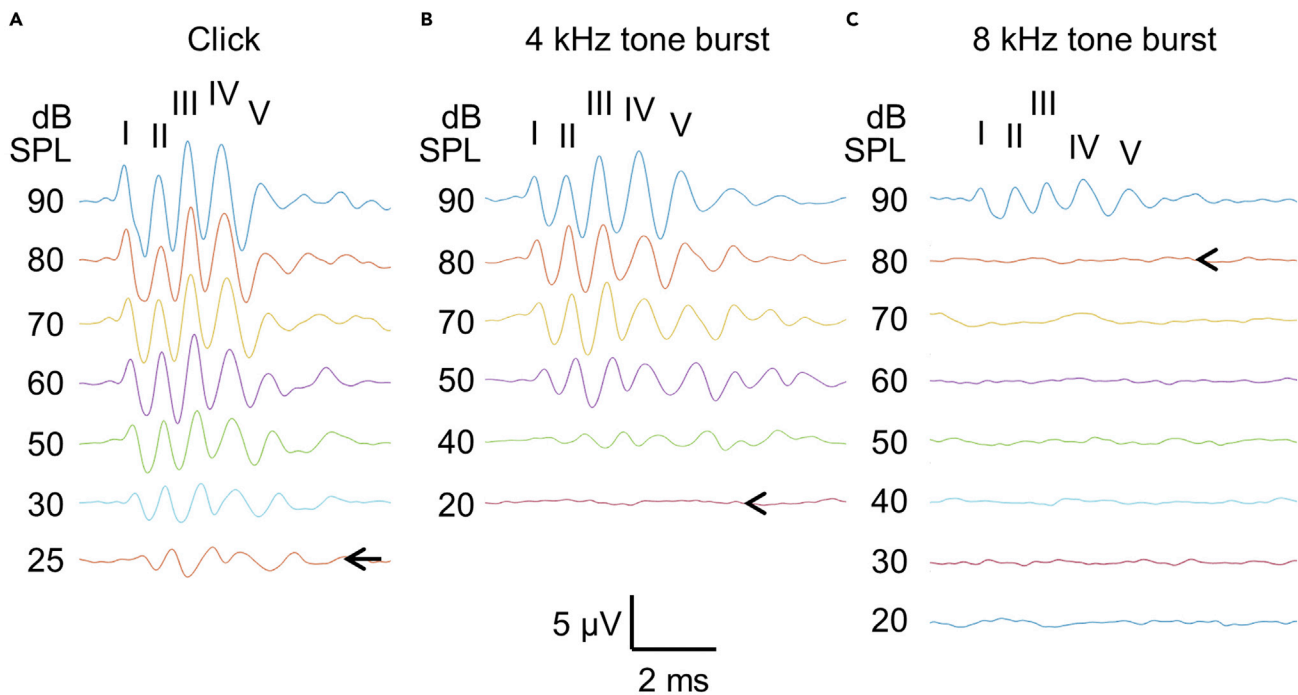


Figure 3. Representative click-evoked ABR recordings under normal condition as well as 4 and 8 kHz tone burst ABR recordings under hypothermic condition

The assayed animals are 8-week old, male C57 BL/6.

(A) Click-evoked ABR recording. Successive waves are labeled with roman numerical I to V. The hearing threshold (arrow) is 25 dB SPL. Peaks II, III and IV are the most robust and stable at low intensities for both click and tone burst stimuli.

(B and C) Hypothermia can have a significant effect on ABR. During the 8 kHz tone burst ABR recording, the mouse appears to be hypothermic. This significantly elevates the thresholds (arrowheads, C).

LIMITATIONS

Whereas it is convenient to use ABR to assess hearing function in mice, this method also has some disadvantages. The rapid rise time of the acoustic stimulus that elicits this response can produce acoustic splatter, which can lead to a decrease in the frequency-specific response. Acoustic splatter usually occurs during the onset and/or offset of transmission, and refers to the spurious emissions owing to an abrupt change in the transmitted signal. Frequency specific ABR can be compromised due to acoustic splatter. Moreover, it is relatively difficult to achieve specific response at low frequency. Importantly, although subsequent changes in the acoustic stimulus waveform can provide important biological information, the ABR does not respond to these alterations. The action potentials associated with neural activity are comparatively small, therefore, ABR results can be affected by interference from feed-through from the speakers emitting acoustic stimuli and other electronic devices in the soundproof chamber. The ABR recording is an objective process, but interpretation of the results is subjective. Determination of ABR threshold relies, at least partially, on the investigator's experience.

TROUBLESHOOTING

It should be noted that the ABR is a field potential that is susceptible to electricity magnetic field, animal anesthesia, loudspeaker and hearing instrument working state.

Problem 1

The connection of the system ([Preparation of the recording equipment](#), step 5).

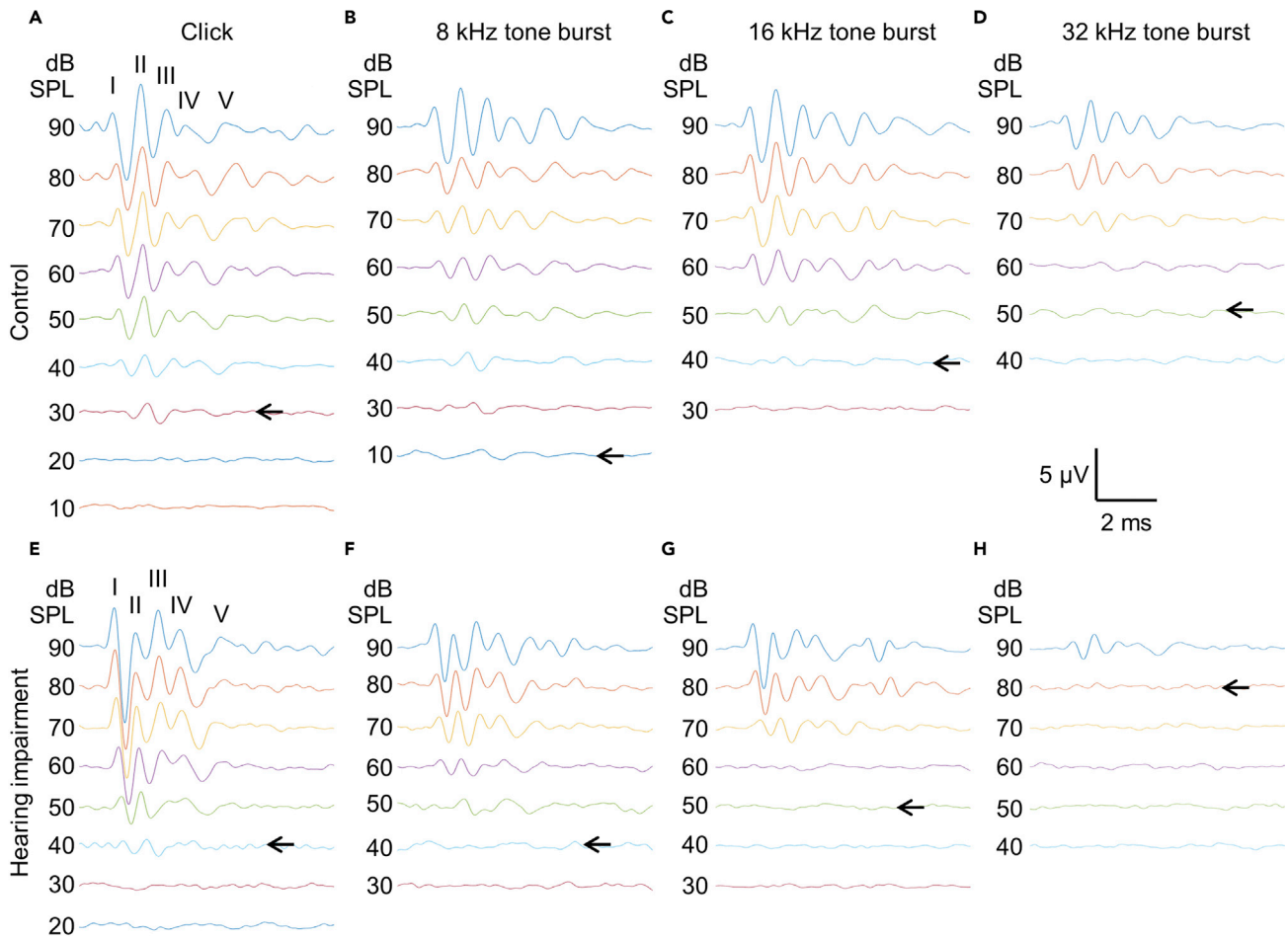


Figure 4. Representative click- and tone burst-evoked ABR recordings of the 12- and 24-week-old C57 BL/6 mice

Successive waves are labeled with Roman numerical I to V. Steps of 10 dB were used in most of the tests.

(A) The click ABR of a 12-week old male C57 BL/6 mice. The threshold (arrow) is 30 dB SPL.

(B–D) The 8, 16 and 32 kHz tone burst-evoked ABR recordings of the 12-week old C57 BL/6 mice. The thresholds, which are indicated by arrows, are 10, 40 and 50 dB SPL, respectively.

(E) The click ABR of a 24-week old male C57 BL/6 mice. The threshold is 40 dB SPL.

(F–H) The 8, 16 and 32 kHz tone burst ABR recordings of the 24-week old C57 BL/6 mice. The thresholds are 40, 50 and 80 dB SPL, respectively.

Potential solution

Following the manufacturer’s instruction, start the Giant ABR program and listen to whether the speaker emits sound. Subsequently, select a channel to plug in the electrode, and touch the electrode with a finger. If the amplitude of this channel changes, it suggests that the connection is intact.

Problem 2

The ABR equipment does not work properly ([Preparation of the recording equipment](#), step 5).

Potential solution

Maintain the instrument in good condition after each assay. Calibrate it regularly to ensure that it will be accurate.

Problem 3

Environmental interference (step 3).

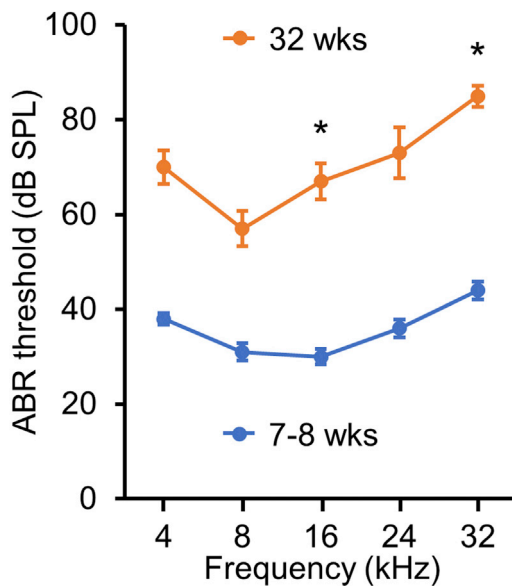


Figure 5. Representative ABR thresholds of male C57 BL/6 mice

The ages of mice were 7–8 and 32 weeks old, respectively. Data are presented as mean \pm SEM. $n=5$ per group. * $P<0.05$, $F(1, 8)=164$ for age; $F(2.41, 19.3)=18$ for frequency, two-way ANOVA with Bonferroni's *post hoc* test.

Potential solution

The action potentials associated with neural activity in the auditory nervous system are small compared with signals from electronic equipment. Therefore, the ABR detection should be carried out in a well-shielded soundproof chamber, and should be far away from electrical noise. Avoiding use of high voltage noise sources, such as refrigerators, freezers or computer monitors, in the recording area. Using the battery packs to power the equipment when recording ABR. Try grounding equipment in the chamber or use aluminum foil for shielding.

Problem 4

Hypothermia (step 3).

Potential solution

Maintain the animal's body temperature at 36.5°C–38.0°C. Hypothermia is a risk factor and has significant effects on the ABR recording. Use a heating pad (e.g., C2Dx TP 700 T/Pump System with temp therapy pad) that does not produce A.C. interference during the procedure.

Problem 5

Electrical interference during ABR recording (step 5).

Potential solution

Turn off electrical equipment in the soundproof chamber during the test. The equipment in the recording chamber can only be powered by the rechargeable battery. Besides, wires connected with the electrodes should be placed away from other wires of the equipment.

Problem 6

Insufficient anesthesia (step 8).

Potential solution

Avertin or a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) are recommended for the anesthesia of mice. If mice show signs of waking up, supplement 1/3 to 1/2 of the original dose of anesthetics and let the animals reach deep anesthesia.

Problem 7

Determination of threshold (expected outcomes).

Potential solution

It is advised that thresholds be determined by a second, experimentally blinded investigator. The data are then compared and differences are resolved.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Guo Zhang (gzhang@hust.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate new data or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

Conceptualization, investigation, and writing – original draft, L.Z.; writing – review & editing, W.Z., D.B., L.X., X.W., and G.Z.; funding acquisition, W.Z. and G.Z.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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