Measurement of the Auditory Brainstem Response (ABR) to Study Auditory Sensitivity in Mice

The ABR is an electroencephalographic response usually measured with scalp electrodes. The electrodes detect voltages produced by neural activity throughout the brain, including the auditory brainstem and eighth cranial nerve. When audible sounds are presented to the ear, they evoke a chain of neural activity that travels up axons and across synapses beginning at the auditory nerve and emanating up through the auditory pathways of the brainstem and forebrain. These relatively minute voltages are buried within a massive amount of other, non-auditory activity in the brain and cannot be detected in this electrophysiologic noise. However, the voltages can be revealed by repeatedly presenting brief sounds and averaging the stimulus-evoked voltages that are time-locked to each acoustic stimulus, with time zero being the onset of each stimulus. The gross voltages are added together as stimuli are presented, with each recording sweep beginning at time zero. The auditory evoked voltages occur at the same post-zero time and are consistent in form for each repetition so they can be superimposed and then summed. Voltages not tied to the stimulus (i.e., the noise) are random with respect to the acoustic stimuli, and after several hundred additions, positive and negative voltages cancel one another, leaving only the auditory evoked response. The response appears as a series of four to five waves, the first occurring ~1 msec after stimulus onset (wave I), the fifth ~5 msec after stimulus onset (see Fig. 8.21B.1). The source of wave I is the auditory nerve, with waves II to V

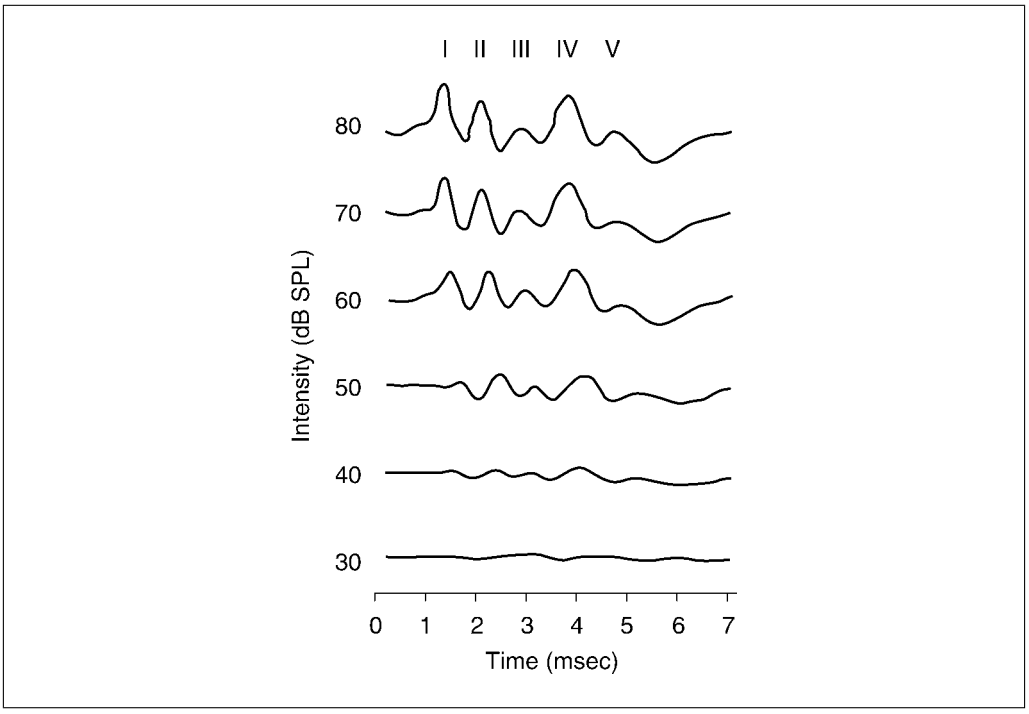

Figure 8.21B.1 ABRs at decreasing intensities. Successive waves are labeled I to V. The wave I voltage arises from the auditory nerve, and waves II through V reflect evoked activity at ascending points in the auditory midbrain. As the stimulus level is reduced, amplitude of ABR waves decrease and latencies of waves increase. Ultimately, no ABR is elicited at the lowest intensity, 30 dB (below threshold). A threshold of 40 dB would be assigned (the lowest intensity at which an ABR can be discerned). Typically, steps of 5 dB are used, rather than 10 dB, as in this simplified example.

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reflecting successively higher levels of the auditory brainstem. For a thorough discussion of ABRs in mice, see Parham et al. (2001).

BASIC PROTOCOL

Before attempting ABR recordings, the equipment must be calibrated so that the desired stimulus intensities are produced during an experiment (steps 1 to 6). Manufacturers' specifications cannot be relied upon. Once the settings are established, periodic testing of the calibration is required. The method described here is for a free-field system (see Alternate Protocol for discussion of closed systems). It is difficult, if not impossible, to measure the sound pressure level (SPL) of the stimuli that the mice will actually be presented with during an experiment because of changes introduced by the measuring device—in this case a microphone. However, in an open system, the effects of the mass of a small microphone are probably minimal.

Thresholds can be obtained in an automated fashion, controlled manually by an observer, or a combination of both, which the author recommends.

In the manual approach, the experimenter has control over which stimuli in the set are presented. This can result in considerable savings in time. For example, the next stimulus level can be presented as soon as an ABR is observed to emerge during the averaging process (this assumes that ABR waveforms are not being stored). In mice, an ABR can be seen emerging after a few dozen stimuli in some cases. The experimenter can also jump to lower intensity levels if desired, and when the threshold level appears near, switch to a greater level of intensity resolution. Moreover, once threshold has been obviously reached, the protocol can be moved to the next frequency.

Automated approaches do not require close monitoring. The ABRs are examined off-line to determine thresholds. The trade-off is time, in that a full set of stimuli are presented unless a sophisticated system that actual determines ABR thresholds is in place. If that is not the case, unnecessary ABRs (e.g., below threshold) will be obtained.

The author recommends a combination approach, whereby automated presentation occurs, but an observer monitors progress and can move to the next stimulus if indicated. It has a measure of efficiency, keeps human involvement during ABR recordings, and reduces experimenter error. Moreover, all ABRs are stored. This allows for thresholds to be determined offline by two or more observers and allows future evaluation of ABR wave amplitudes and latencies.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must follow officially approved procedures for the care and use of laboratory animals.

Materials

Mice

Appropriate anesthetic (e.g., Avertin or a combination of ketamine·HCl and xylazine i.p.; *APPENDIX 4B*)

Sound-attenuated, electrically shielded chamber or box for recording (e.g., Industrial Acoustics Corporation)

Sound-calibrating equipment: high-quality sound measuring devices having high-frequency microphone and ability to measure impulse stimuli (e.g., Bruel & Kjaer)

Clamp and stand

Equipment and software for ABR recording and stimulus presentation designed for use with rodents (e.g., Tucker-Davis Technologies; Intelligent Hearing Systems)

Speakers

Computer to run recording and stimulus-generating equipment (see manufacturer requirements)
Oscilloscope, optional
Large, soft-grip forceps for handling mice (check with local veterinarians for policy), optional
1-ml (for Avertin) or 100-μl (for ketamine/xylazine) syringes
23- or 25-G needles
Heating lamp or pad (e.g., Frederick Haer) with appropriate enclosure (e.g., cage)
Foam rubber
Needle scalp electrodes (thinnest available, e.g., 29-G; *http://www.electrodestore.com*)
Small, curved forceps to hold scalp while inserting needle electrodes

Calibrate equipment

The calibration check described in steps 1 to 6 does not have to be performed on every mouse but should be done periodically, such as the first mouse of the day.

1. Place the calibrating microphone in a space adjacent to the outer ear of an anesthetized mouse, just below the ear canal so as not to block it or the pinna. Hold in place with a clamp on a small stand or similar device.

   An anesthetized mouse can be used to calibrate the system initially.

   The microphone is typically aimed at the speaker, but directionality of the microphone should be ascertained from the manufacturer's specifications.

   Commercially available speakers and headphones are adequate for routine ABRs. Earphone or tweeter specifications sometimes extend well above 20 kHz and should be evaluated. However, manufacturers may provide broad-band, ultrasonic speakers with the ABR equipment.

   Laboratories using ABRs as an ancillary technique may not have such equipment. One solution is to borrow the equipment, and make accurate calibrations. These may be referenced against readings made using a less-expensive device. For day-to-day use, the lesser equipment is adequate for assuring that the acoustics have not undergone a general change in output.

2. Instruct the software to present each stimulus at a nominal intensity (70 dB SPL) and measure the actual output to the calibrating microphone. If the actual intensity differs from that intended by the software, adjust the attenuation in the ABR software.

   It is advisable to check that the system's attenuation performance is accurate. That is, once the system is producing a 70-dB tone, instruct it to attenuate the tone by 10 dB and make sure that the result is 60 dB.

   Sound levels are measured in decibels (dB), and when referenced to the standard intensity of 20 μPa (this is equivalent to 0.0002 dynes/cm^2 or 20 μN/m^2 in older literature), the term dB SPL (sound pressure level) is employed. To be calibrated, each stimulus (tone bursts at each frequency, noise bursts, and/or click) must be sufficiently intense (e.g., 70 dB SPL) to be differentiated from the ambient noise measured by the meter in quiet.

3. Make several measurements at slightly different locations at the outer ear to provide an assessment of the variability of the sound field. Choose the setting that best represents the mean intensity at the outer ear.

   Because the temperature, humidity, and other conditions will normally vary little in the laboratory, the sound system should be relatively stable from day to day. Nonetheless, it is important to periodically check the system.

4. To establish values for day-to-day calibration, remove the mouse after performing the above procedures. Check the calibration for each stimulus at 70 dB SPL (these may differ slightly because of the now-absent influence of the mouse's head and body). Note any discrepancies.

For subsequent periodic checking of the sound system, use these without-the-mouse values. In this way, one does not have to have a mouse in the box to check the calibration.

5. Monitor the output of the sound-measuring device on an oscilloscope to confirm that the sound meter can react rapidly enough to measure the stimulus (optional); the waveform should conform to the expected output.

   ABR stimuli (especially clicks) are very brief and the sound-measuring device should have an impulse function for measuring clicks and brief tone or noise bursts. One way to calibrate brief tone or noise bursts is to elongate their duration during calibration so the meter can achieve a steady response. A 3-msec tone burst used during ABR recording is likely to achieve the same level during the "plateau" between rise and fall.

6. Return the calibrating microphone to the position established during the initial calibration of the system.

   The calibration will normally not vary significantly. However, if variation is detected, it will be necessary to troubleshoot to determine the source of the problem.

Check acoustic calibration

7. Position the microphone in the established standard position determined in the calibration (see steps 1 to 6) and check the 70-dB SPL values.

   This check may be done just once on the day of recording.

Anesthetize mouse

8. Load syringe with anesthetic. Consult the institutional veterinary staff regarding approved anesthetics and dosages, which must be specified in the animal care and use protocol.

   Relatively short-acting, safe anesthetics include ketamine/xylazine and Avertin (tribromoethanol). Avertin is delivered at a rate of ~500 mg/kg; ketamine/xylazine at 80 to 100 (ketamine)/5 to 10 (xylazine) mg/kg body weight (consult local veterinarians for policy).

9. Use one of the following two methods of administering intraperitoneal injections to mice:

a. Grasp the mouse by the tail, suspend it over its cage perpendicular to the cage bars (head down), and allow it to grasp a bar while applying light tension by pulling on the tail. Gently grasp the loose skin behind the shoulders with the thumb and forefinger of other hand, using the pinky to secure the tail so that the abdomen is taut. Insert the hypodermic needle into the belly and inject.

b. An alternative method requires less handling and dexterity. Grasp the tail and invert the mouse (ventral side up). With the tail outside the edge of the cage and the body inside the cage, gently lower the cage top without pinching the tail, exposing the belly between the cage bars (the tail and experimenter's hand holding it are outside the cage). Guide the syringe needle through the bars and inject the mouse.

   Experimenters who have not anesthetized mice are urged to obtain training from a veterinary staff or other experienced persons.

Implant electrodes

10. Place the anesthetized mouse in a fixed position within the recording chamber (the same as that used to initially calibrate the sound system). Place the body on a heating pad and face the head towards the speaker 18 cm away (>2 wavelengths at a lowest frequency of 4 kHz). Elevate the mouse's chin slightly and cradle it with a piece of foam rubber so it does not move from side to side while still allowing the mouse to breath easily.

11. Keep anesthetized mice warm (e.g., near 38°C) before, during, and after ABR recordings. Before and after recordings, use a heating pad or incandescent light above the

cage. In either case, take care not to overheat the animal. During ABR recordings, use a heating pad that does not produce A.C. interference.

Mice become hypothermic very easily when anesthetized, and this can have significant effects on the ABR (Henry, 1979; Williston and Jewett, 1982; Shaw, 1986).

Direct current heating pads are commercially available. Alternatively, hot-water circulating systems can be used. A rectal thermal probe is recommended to monitor and adjust body temperature during recording. It is also acceptable to calibrate the temperature maintenance system initially using a probe to determine the approximate settings necessary to maintain temperature. The latter may be preferable when small or immature mice are tested.

12. Place the active electrode into the scalp between the ears (over the vertex of the skull), with a reference electrode near one cheek and a ground electrode at the other cheek or tail. Use small, curved forceps to grasp a fold of skin. Make sure that the electrode sits without tension.

    A subdermal needle electrode is most advantageous for use in mice and appears to result in no discernable damage to the scalp. Commercially purchased, small electrodes for use in humans work well. While it is possible to surgically implant electrodes on the skull and test mice after recovery while unanesthetized, this is technically difficult and not suitable for obtaining thresholds or consistent ABR measures. The movements of the animal make for an uneven sound field and generate voltage artifacts. Thus, general anesthesia is usually employed.

Present stimuli and record ABRs

13. Present brief tone stimuli (e.g., 1 msec rise-fall and 3 msec duration) at a rate of ~20 stimuli/sec.

    The evoked response requires a 10-msec window of recordings (beginning at sweep time zero). This allows the ABR, which spans ~1 to 6 msec (Fig. 8.21B.1), to be visualized.

14. Obtain thresholds in either an automated fashion, one controlled manually by an observer, or a combination of both. To carry out the recommended combination, proceed as follows:

    a. For each ABR, present the stimulus 300 to 500 times.

       For human recording, 1000 repetitions are typically required to resolve high-quality ABRs. With mice, however, this number is much smaller, with clear ABRs often emerging after <100 repetitions. Whereas 300 repetitions for each stimulus are more than adequate in most cases, the experimenter may find that a smaller number of repetitions provides clear, reliable ABRs.

    b. Begin with a relatively intense SPL for a given stimulus (e.g., 80 dB SPL); obtain an ABR, and reduce the intensity until no ABR can be discerned.

       The resolution with respect to intensity steps must be determined; 5-dB steps represent a reasonable interval.

       It is not practical or reliable to use 1-dB steps; thus, most researchers choose final steps of 5 dB SPL, with threshold determined to the nearest 5-dB step.

    c. Set the automated program (software provided by the manufacturer) to record ABRs at 5-dB steps from 80 dB SPL to 10 dB SPL (a level that will be below threshold for most normal-hearing mice).

       The 10-dB default value can be adjusted with experience. If 10 dB is found to be above the threshold for some mice, lower intensity steps can be added. Or, with mice known to have hearing loss, low intensity steps can be eliminated (e.g., if it is well established that no mice of a hearing-impaired strain have thresholds below, e.g., 50 dB, it is not necessary to test lower intensities). To save time, have the initial, higher intensity steps (well above threshold) at 10 dB, rather than 5 dB. Set the equipment

so that the electroencephalographic response is displayed as an average as the ABR is emerging. For each stimulus in the protocol, run the entire 300+ repetitions and store the ABR. At some decreasing intensity step, it may be clear that an ABR is no longer present (threshold has been reached). The protocol can then be manually moved to the next stimulus and the procedure repeated. With this method, all ABRs are stored on the computer for off-line evaluation. Many variations of this approach can be used, ranging from complete automation to complete manual control, where all ABRs are not stored and the experimenter chooses to skip some stimulus levels. For instance, the experimenter can begin with levels near threshold if the likely threshold range of the mouse being tested is known (e.g., for a known inbred or mutant strain).

d. Monitor the ABRs during or after the recordings and determine if any stimuli need to be repeated (e.g., if the ABR waveforms are abnormal or artifacts are present).

15. Monitor the condition of the mouse. If it begins to move and generate movement artifacts, suspend the program and administer supplemental anesthesia at a rate of one-third to one-half the initial dose (consult institutional veterinarian for local policy). Resume ABR testing when anesthesia has taken effect.

16. Upon completion of protocol, remove the mouse, check its respiration, place it in a warm recovery setting, and monitor until recovered from anesthesia.

Analyze data offline

17. Determine the following ABR parameters: amplitude (voltage) of waves, latency (time elapsed from time zero to wave peak), inter-peak latency (an indication of transmission time), and threshold.

Threshold is the most important variable for assessing auditory sensitivity and is the focus of this unit. The approach is straightforward and conceptually similar to methods for obtaining any threshold: stimulus intensity is decreased from a maximum (e.g., 80 dB SPL) in steps, and ABRs are obtained at each intensity. Threshold is defined as the minimum intensity at which a reliable ABR can be obtained. In Figure 8.21B.1, a discernable ABR is evoked by the 40-dB SPL stimulus but not by 30-dB stimulus; therefore, threshold would be defined as 40 dB. Most procedures would then use intermediate intensities (e.g., 5-dB step) to obtain a higher resolution of threshold.

18. Perform an analysis of variance to compare ABR threshold changes within subjects or between groups.

Factors may include subject group (independent measure) or subject age (repeated measure), and stimulus frequency (repeated measure). Results are typically presented graphically with stimulus intensity (of threshold) as the ordinate and stimulus frequency as the abscissa. Threshold should be referred to as ABR threshold, not threshold for hearing.

ALTERNATE PROTOCOL

ABR RECORDING IN A CLOSED SYSTEM

There are two basic methods for delivering acoustic stimuli for ABR recordings: (1) free-field systems in which a speaker is placed some distance from the animal (as recommended and described in the Basic Protocol), and (2) closed systems, in which sound is delivered via a tube or probe inserted into or coupled to the ear canal (Henry, 1979). Closed systems have been widely used in auditory physiological research on larger animals such as cats and guinea pigs, but present several problems with mice. It is difficult to present sounds through a small-bore tube coupled to the mouse's ear (probes inserted well into the ear canal are especially problematic) and to maintain a consistent tube-ear coupling from mouse to mouse. The problem of high-frequency standing waves must also be considered (minute dead and enhanced loci within a closed space). Calibrating a closed system is problematic because the impedance of the ear should ideally be replicated to interface with the calibrating microphone (which itself has a much larger diameter than the mouse ear canal). Moreover, coupling must be consistent, and this can be hard to achieve with a small animal. At the least, a microphone should be placed at the sound

tube opening to measure output in order to calibrate the system to some nominal standard. At best, an artificial ear that approximates the mouse external ear while accommodating the calibrating microphone can be fashioned.

Another issue with respect to a closed system is the fact that the auditory system begins at the outer ear, including the pinnae. The use of a coupled tube essentially bypasses the effects of the pinnae and head, and does not take into account the effects of these structures on hearing, such as resonance and head shadow (qualitative differences are evident to anyone who has listened through an insert earphone or tube stuck into the ear).

To obtain monaural responses, a sound tube might be used. Occlusion of one ear with a viscous fluid such as glycerol can also be used as a monaural preparation in a free-field system. However, it is only practical to occlude one ear during a recording session because the occluded ear would have to be cleared. A caveat for monaural testing is that, because of the small size of the mouse's head, the inter-aural attenuation is relatively small. Thus, suprathreshold stimuli are likely to stimulate both ears, even with monaural delivery.

COMMENTARY

Background Information

Threshold determination

There is no such thing as a true ABR threshold that generalizes across laboratories or testing situations. The thresholds obtained depend on the particular recording and sound generation setup, calibration of the sound system, and point at which the sound pressure level is measured (e.g., outer ear versus eardrum).

Regardless of the sound delivery system used, consistency is of the utmost importance. For most applications (e.g., evaluation of mutations or age-related changes), thresholds with respect to a consistent control or standard are most useful. For example, it may matter little if normal thresholds of one laboratory differ from those of another laboratory by 5 to 10 dB, as long as the degree of hearing loss is accurate and consistent within the laboratory. Thus, the sound field or tube must be regularly calibrated. Moreover, normal-hearing mice should be regularly tested as a biological calibration. Finally, the human ear should not be overlooked as a rough test of the lower frequencies as an indicator of sound system change, malfunction, or distortion.

What constitutes the threshold?

Psychophysical threshold is formally defined as the minimal level at which a response is elicited by at least 50% of stimulus presentations. This is not feasible for ABRs because each ABR is constructed from numerous, averaged, stimulus-response repetitions; there is no way of knowing how many sweeps evoked the minute responses that contribute to the final average. Thus, the operational definition of threshold is the minimum stimulus intensity that elicits an observable ABR response. While seemingly straightforward, qualifications or caveats need to be included (the following assumes that the software does not include a sophisticated algorithm for automated threshold determination).

What sort of waveform is indicative of a response? Waves I through V are not uniform in clarity, and the relative clarity of individual waves may change as stimulus intensity is altered. A common solution is to use any wave(s) clearly discernable at the minimum intensity. In mice this is often wave I, but this can vary depending on electrode configuration or other factors. It can be problematic to tell the difference between a minimal ABR wave and non-auditory voltage bumps in the record. This issue can be addressed in several ways. First is to determine whether the wave can be replicated: a second ABR at or near the threshold level that produces an overlapping waveform suggests a non-random event (most programs allow one to visually overlap the waves from several recordings). Second is to use an operational definition based on a clearly discernable waveform. This may produce slightly higher thresholds, but if a consistent criterion is used for all mice, this is not a drawback. Third is to examine the ABR records as intensity is decreased to threshold levels. Waves are very clear at suprathreshold levels, and one can watch the waves diminish in the records as intensity decreases. If a wave does not disappear with reducing levels, but persists in an unchanging form, it is probably an artifact.

It is strongly recommended that thresholds be determined by a second, experimentally blind rater who examines the ABR records. Thresholds are then compared and any differences resolved.

What constitutes the normal hearing threshold for mice?

Some researchers choose the CBA/J or CBA/CaJ strains as "normal" strains, because thresholds are relatively low and the mice retain fairly good hearing well into the second year of life. It is the author's view that the term "normal thresholds" should not be used to refer to mice in general, because strains often differ. For example, young C57BL/6J mice at the peak of their hearing can have lower thresholds for low frequencies than CBA strains (Willott, 1986). Neither strain is more or less representative of mice in general. When evaluating a procedure, treatment, or genetic manipulation that may alter hearing, it is most important to use control mice of the same strain, age, and/or sex as the "normal" reference.

ABR thresholds and hearing thresholds

Whereas the ABR threshold is used to assess auditory sensitivity, it is not actually the threshold of hearing. As mentioned earlier, the ABR measures physiological activity associated with hearing. While closely related to psychophysical hearing thresholds, there are typically some differences. These are presumably due in part to differences in the properties of stimuli used in each approach. ABR stimuli are brief with a rapid rise-fall time, and this causes a degree of frequency spectrum splatter when tone bursts are used; by contrast, behavioral or psychophysical stimuli can be long and have a gradual rise-fall, resulting in a purer frequency spectrum. The brevity of ABR stimuli can also elevate thresholds (so-called time-intensity trading). Also, ABR stimuli are presented very rapidly (e.g., 10 to 20 Hz or more), whereas this is not a requirement for behavioral thresholds. Thus, tone stimuli are qualitatively different for ABR and behavioral experiments. Moreover, mice are anesthetized during ABR recordings, which is obviously not the case for a behaving mouse. Given all this, it should properly be stated that an ABR threshold is used to estimate or evaluate hearing sensitivity—terms that do not imply equivalence. Therefore, "normal" ABR thresholds generally indicate normal hearing sensitivity, and elevated ABR thresholds indicate elevated hearing thresholds (assuming there are no factors that degrade the ABRs per se, such as neurological changes that would disrupt the synchrony of evoked discharges, synaptic transmission, or axon physiology).

Critical Parameters

Equipment

It is recommended that equipment designed to acquire ABR recordings from mice be used. Tucker-Davis Technologies provides a very flexible system with many capabilities besides ABR recording and also manufactures an excellent electrostatic driver with a good frequency response approaching 100 kHz. The disadvantage in its flexibility is that setting up ABR recordings is more difficult than with a dedicated ABR system. Intelligent Hearing Systems provides a simpler system without a wide range of functions.

Test environment

The mouse and speakers should be located within a box, chamber, or room that is both electrically shielded and sound-attenuated. These can be purchased from commercial suppliers or custom made in-house. Sound attenuation is relatively easy for mouse recording because one need not be concerned about low frequencies (e.g., <1000 Hz) that are inaudible to mice. Moreover, a relatively small chamber can be made anechoic for high frequencies (for a free-field sound system) using foam rubber sheets with bumpy surfaces, such as that used for mattress pads.

EEG parameters

Commercially available ABR recording setups come with hardware and software to establish recording parameters. Voltages must be both amplified and filtered. The amplifier gain should be set to produce easily viewed ABR waves, but the gain used is one of experimenter preference. Absolute ABR amplitude probably has little physiological relevance because it is affected by many variables such as impedance of the electrode, electrode placement, scalp properties, and anesthesia. By trial and error, the gain should be set so as not to over-amplify noise voltages when an ABR is not present (e.g., below threshold), while making small ABRs discernable. It is important to maintain a record of amplifier gain so that ABRs can be compared across mice to detect changes in a system or unusual ABR amplitudes.

Filter settings in most studies of mouse ABRs have used a low-pass of 3000 Hz and a high-pass of 100 to 300 Hz. The 300 Hz high-pass setting produces a flatter baseline by

attenuating line voltages or other low-frequency fluctuations (e.g., breathing artifacts), but also makes ABR waves less sharp.

Stimulus parameters

Because ABRs are evoked by the initial segment of the acoustic stimuli, brief tone bursts or clicks are used. The rise time needs to be rather abrupt so that repeated evoked potentials are synchronized for averaging. This results in some splatter of the frequency spectrum of tone bursts, with less frequency-specific stimuli. A 1-msec rise-fall time and 3-msec duration is a reasonable compromise (a zero rise time would produce acoustic click artifacts). Because high frequencies are used, this is less of a problem with mice. Even a relatively low frequency of 4 kHz has four cycles within a 1-msec rise-time. Clicks have a broad frequency spectrum that is a function of duration of the pulse, sound production equipment, and speakers.

Tone bursts. The number of tone frequencies used depends on the purpose of testing. For high-throughput screening, a minimal number is advantageous. The author recommends at least three tones (low, middle, and high) because hearing loss is often not linear across frequencies. Octaves of 8, 16, and 32 kHz are usually adequate. For a more comprehensive test, more frequencies are required (e.g., 4, 8, 12, 16, 24, and 32 kHz). For work done on mice with normal hearing, even higher tones can be added (e.g., 48 or 64 kHz if possible).

Clicks. Click stimuli can be used for the rapid, general assessment of hearing. The frequency spectrum of clicks is broad, although it is affected by the duration of the voltage pulses sent to a speaker as well as the audio system. Standard click parameters should be chosen and used for all experiments. Broadband noise bursts can also be used for general assessment (as with tones, a 3-msec duration and 1-msec rise-fall). Because of their broad frequency spectrum, clicks and noise bursts may not allow detection of hearing loss restricted to high or low frequencies because the intact regions of the cochlea can still respond and have low thresholds. Thus, the use of clicks or noise bursts alone is not recommended for the comprehensive evaluation of hearing.

Maximum intensity levels. An issue in many studies, especially those involving mice with elevated thresholds, is the choice of maximum intensity level to use in the ABR protocol. The author has typically used a maximum of 80 dB SPL, assigning a nominal threshold of 85 dB when ABRs cannot be obtained. The author's opinion is that clicks or tone pulses of >80 dB SPL are likely to be acoustically distorted as well as biologically irrelevant. A mouse that cannot hear a tone at 80 dB SPL can be considered severely impaired for that frequency. Moreover, very high intensities are likely to produce acoustic distortions and affect cochlear mechanics in nonbiological ways. However, some investigators use intensities as high as 100 dB SPL.

Repetition rate. Stimuli can be presented rapidly without affecting the ABR, as long as responses to successive stimuli do not overlap or affect one another. The author typically uses a repetition rate of ~20 Hz (software may set this at a slightly different rate). Although faster rates can be used, the rate can affect the ABR of older mice and perhaps certain hearing-impaired mice.

Artifact rejection

Commercially available recording systems such as those mentioned in the Basic Protocol have an artifact rejection function that prevents a sweep from being recorded when a voltage exceeds a certain size. The rejection voltage threshold is typically set by trial and error so that neither too many nor too few artifacts are rejected. The occurrence of numerous artifacts is usually indicative of some problem, such as poor electrode placement, animal movements, or electrical interference. The problem should be fixed, if possible, since a high level of artifact rejection is not optimal for obtaining good recordings.

Troubleshooting

Acoustics

Audio systems may produce unwanted acoustic distortions when their intended limits are exceeded. Clicks, swooshes, or other unwanted sounds can sometimes be emitted from speakers, especially when ultrasonic stimuli are produced at higher intensities. Here, the human ear is an excellent detector, since tones ≥20 kHz should not be audible. It is also beneficial/advantageous that the output of the calibrating microphone be monitored on an oscilloscope. This allows distortion or artifacts in the acoustic waveform to be observed.

Another issue is the consistency of the sound system. It should be regularly calibrated and, if the output differs from the expected calibrated values, system components (speakers, etc.) should be checked. If necessary, re-calibration should be performed and the system adjusted.

Shielding

Because the voltages produced by neural activity are so small, they must be greatly amplified during recording, typically using a preamplifer placed near the animal. This unfortunately also amplifies two types of unwanted voltages: feed-through from the speakers emitting acoustic stimuli (see below) and 60 Hz A.C. line voltages. Both types of voltages will be picked up by the recording electrode and contaminate the evoked potential. Thus, proper electrical shielding and grounding are important to eliminate the interference. This is done by encasing the speaker in a metal case or screening that is grounded. Speaker cables must also be shielded and grounded. The 60-Hz interference can be reduced using a notch filter, but best results are obtained by eliminating it with shielding and grounding and by having no A.C.-powered equipment in the recording chamber. It may be necessary to ground everything to a ground post on (or common to) the recording equipment to avoid ground loops. Sometimes trial and error is required to find the most suitable grounding configuration.

Feed-through is usually easily distinguished from actual neural activity. Speaker feed-through will exactly mirror the electromagnetic signal of the speakers producing no delays (latency) in the recording. The 60-Hz waveform is distinctive as well. One low-tech method of determining if recordings are contaminated by artifacts is to confirm that they disappear if a mouse dies. Thus, if an animal is slated for euthanasia, ABRs can be monitored during this process. Artifact voltages will persist after the mouse has died. This determination would require approval from the institutional animal care and use committee.

Anticipated Results

Table 8.21B.1 presents representative ABR thresholds from eight control (untreated),

Table 8.21B.1 An Example of Typical ABR Thresholds of Individual Mice with Means and Standard Errors[a]

Control group						
Frequency (kHz)	4	8	12	16	24	32
	20	35	25	40	65	85
	35	35	30	45	75	85
	30	35	35	50	85	85
	25	45	30	55	70	85
	40	45	70	65	80	85
	25	30	35	45	60	85
	25	15	20	30	20	85
	25	20	30	20	45	85
Means (SEM)	28(2)	33(4)	34(5)	44(5)	63(8)	85(0)
Experimental group						
	25	25	30	30	25	65
	25	35	25	15	15	75
	10	30	25	30	30	80
	30	25	25	30	40	75
	35	25	20	35	70	85
	30	40	25	35	75	70
	15	30	15	15	30	70
	20	20	10	10	35	65
Means (SEM)	24(3)	29(2)	22(2)	25(4)	40(8)	73(2)

[a]Data from Willott and Turner, 1999

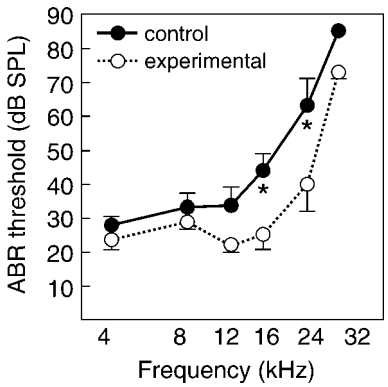

Figure 8.21B.2 An example of the means and SEMs for ABR thresholds of 6-month-old C57BL/6J mice (data from Table 8.21B.1). Filled circles indicate control mice; open circles are mice treated with exposure to an AAE. Thresholds in the latter group are generally lower than those of controls, indicating an ameliorative effect on ABR thresholds. Asterisks indicate significant differences as determined from Tukey-Kramer tests for paired comparisons. Note that frequency (abscissa) is a logarithmic scale.

6-month-old C57BL/6J mice and eight like-aged experimental mice. The purpose of the experiment was to investigate the effects of sound exposure on progressive hearing loss. The experimental mice were exposed every night to an augmented acoustic environment (AAE), 70-dB SPL noise bursts, from age 25 days. Control C57BL/6J mice (filled circles) exhibited strain-typical, age-related elevation of ABR thresholds for tones >12 kHz (at 1 month of age thresholds are between 20 and 30 dB from 12 through 32 kHz; Fig. 8.21B.2). Exposure to the AAE had an ameliorative effect (open circles), in that thresholds for high frequencies remained relatively low compared to controls.

The data were analyzed using a two-way mixed ANOVA (2×6), with group (2) as an independent measure and frequency (6) as a repeated measure. The main effect of group was significant [$F(1, 14) = 8.36$, $p = 0.012$], as was the main effect of frequency [$F(5, 70) = 72.2$, $p < 0.0001$], and the group $\times$ frequency interaction [$F(5,70) = 2.42$, $p = 0.046$].

The group effect reflects the generally lower thresholds across frequencies in the experimental group, whereas the interaction appears to be due to the relatively higher thresholds at 16 and 24 kHz in controls. A follow-up test comparing means (e.g., Tukey-Kramer test) shows that, indeed, the mean thresholds differ significantly ($p < 0.01$) for 16 and 24 kHz only.

Time Considerations

The time required to determine a set of ABR thresholds will depend on the number of stimuli used. For rapid screening (e.g., clicks and three tone burst frequencies) with 300 repetitions at a rate of 20 Hz: each ABR acquisition requires 15 sec. If the typical threshold were 25 dB SPL and the starting point was 80 dB SPL followed by twelve 5-dB steps, the time required to determine the threshold would be ~12 min per mouse. The overall experimental time can be reduced by using 10-dB steps above threshold and 5-dB steps only near threshold. For example, the software can be set to present levels of 80, 70, 60, 50, 45, 40, 35, and so on, when the threshold of mice tested is typically <50 dB SPL. Also, the experimenter can manually change from 10-dB steps to 5-dB steps as required. This can reduce the experimental time by about one-half. Some researchers may find that 200 repetitions provide sufficient quality ABRs for screening, further reducing time. If time is not of the essence, a thorough set of ABRs can be obtained in ~30 min.

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Key References

Parham et al., 2001. See above.

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Provides a thorough discussion of the ABR in mice.

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