Using the Auditory Brainstem Response (ABR) to Determine Sensitivity of Hearing in Mutant Mice

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ABSTRACT

Measurements of auditory evoked potentials can be used to determine reliably an audiometric representation of hearing sensitivity in mice. In a high-throughput phenotyping screen of mice carrying targeted mutations of single genes, the auditory brainstem response (ABR) is used to gain an estimate of hearing threshold for broadband click stimuli and pure tone frequencies ranging from 6 to 30 kHz. Comparison of thresholds obtained in mutant and wild-type mice give a means to determine mild, moderate, and severe hearing impairment. This gives a clear advantage over using a "clickbox" test to assess hearing by observations of the Preyer reflex. The ABR screen has identified several mutant lines with mild and moderate hearing loss, which appear to demonstrate normal Preyer responses. The ABR technique also allows frequency-selective hearing loss to be identified. *Curr. Protoc. Mouse Biol.* 1:279-287 © 2011 by John Wiley & Sons, Inc.

Keywords: hearing • evoked potential • electrophysiology • high-throughput screening

BASIC PROTOCOL

The auditory brainstem response (ABR) is an established method for determination of hearing sensitivity in patients in the clinic and in animal studies of hearing loss in the laboratory. Evoked electrical potentials recorded from the scalp are averaged to produce a physiological waveform representing auditory neural activity in the brainstem and can be achieved using a wide variety of equipment. In human testing, it is common for a very high number of potentials to be averaged to generate a recognizable ABR waveform. In animal studies, the use of anesthesia to sedate and immobilize the animal reduces the magnitude of biological/myogenic noise in the recording system and facilitates fewer record sweeps to be required to produce a clear averaged waveform.

Here, a custom hardware and software system for high-throughput ABR studies is described. It is typical to record click-evoked and tone-evoked ABRs across a range of sound pressure levels to determine the ABR threshold for each stimulus. Using tone pips of 6, 12, 18, 24, and 30 kHz allows an audiometric profile for each mouse tested to be constructed and an average audiogram for each mutant mouse line to be generated and compared with a baseline dataset of wild-type mice on the same genetic background. This approach has been used to screen over 330 lines of mutant mouse. Phenotyping results can be viewed online at *http://www.sanger.ac.uk/mouseportal*.

NOTE: All protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals [e.g., the guidelines of the UK Home Office Animals (Scientific Procedures) Act 1986].

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Materials

Male or female mice: control and mutant, tested at an appropriate age (no ABRs are likely to be recordable before the onset of hearing at around postnatal day 12)
Ketamine/xylazine anesthetic (see recipe)
Viscotears (liquid eye gel containing 2 mg/g Carbomer, with cetrimide; Dr Mann-Pharma)
Atipamezole mix (recovery agent; see recipe)

Sound-attenuating chamber (e.g., Industrial Acoustics)
Heating blanket (to prevent hypothermia in the mouse)
Stimulus-generation and calibration equipment (Tucker-Davis Technologies, TDT) including:
RP2.1 Enhanced Real-time processor PA5 Programmable Attenuator
SA1 Stereo Amplifier
ACO Pacific Microphone (7017) and Preamplifier (PS9200) for calibration*
MA3 Stereo Microphone Amplifier
Sound Transducer (CTS Type 341; RS Components part no. 172-7712)*
BNC and other connector cables
1-ml syringes (for injection; BD Plastipak)
27-G, 13-mm length hypodermic needles (for injection; BD Microlance)
Response-processing equipment (Tucker-Davis Technologies) including:
Needle electrodes (Chalgren Enterprises, cat. no. 112-812-48-TP; disposable low-profile EEG needle electrodes; Fig. 1A)
Low-Impedance Recording Headstage/Preamplifier (RA4LI + RA4PA)*
RA16 Medusa Base Station
RP2.1 Enhanced Realtime processor
HB7 Headphone Buffer (Optional)
MS2 Monitor Speaker (Optional)
BNC and other connector cables
Personal computer, housing TDT gigabit interface, TDT driver software and bespoke averager software (available on request)
Digital Oscilloscope (to view stimulus and electrode signals; optional)

NOTE: Items with an asterisk (*) next to them are housed within the sound attenuating chamber.

Calibrate the sound system

1. Amplify and digitize (RA4LI and RA4PA) the electrode signals before returning them via an optical link to the RA16 basestation for filtering and then to the second RP2.1 for sampling at 100 kHz. An optional connection from the RA16 basestation, via the HB7 headphone buffer to the MS2 monitor speaker gives an audible output of the electrode signal to provide a very useful means of monitoring the condition of the animal.

   The signal allows the ECG of the mouse along with baseline myogenic activity and breathing rhythm to be heard. Synchronized triggering of stimulus presentation and recording allows short samples (20 msec) of electrode activity to be averaged under software control to generate the ABR-evoked potential waveform trace. It is not necessary to use artifact rejection (e.g., to reject sweep records containing an ECG component).

Anesthetize the mouse and prepare for recording

2. Anesthetize the mouse with a 10 ml/kg intraperitoneal injection of ketamine/xylazine (0.1 ml/10 g body weight containing 10 mg ketamine/10 g body weight and 0.1 mg xylazine/10 g body weight). Once the mouse is immobilized, place it on the heating blanket in a sound-attenuating chamber and add a drop of Viscotears to each eye to prevent drying of the cornea.

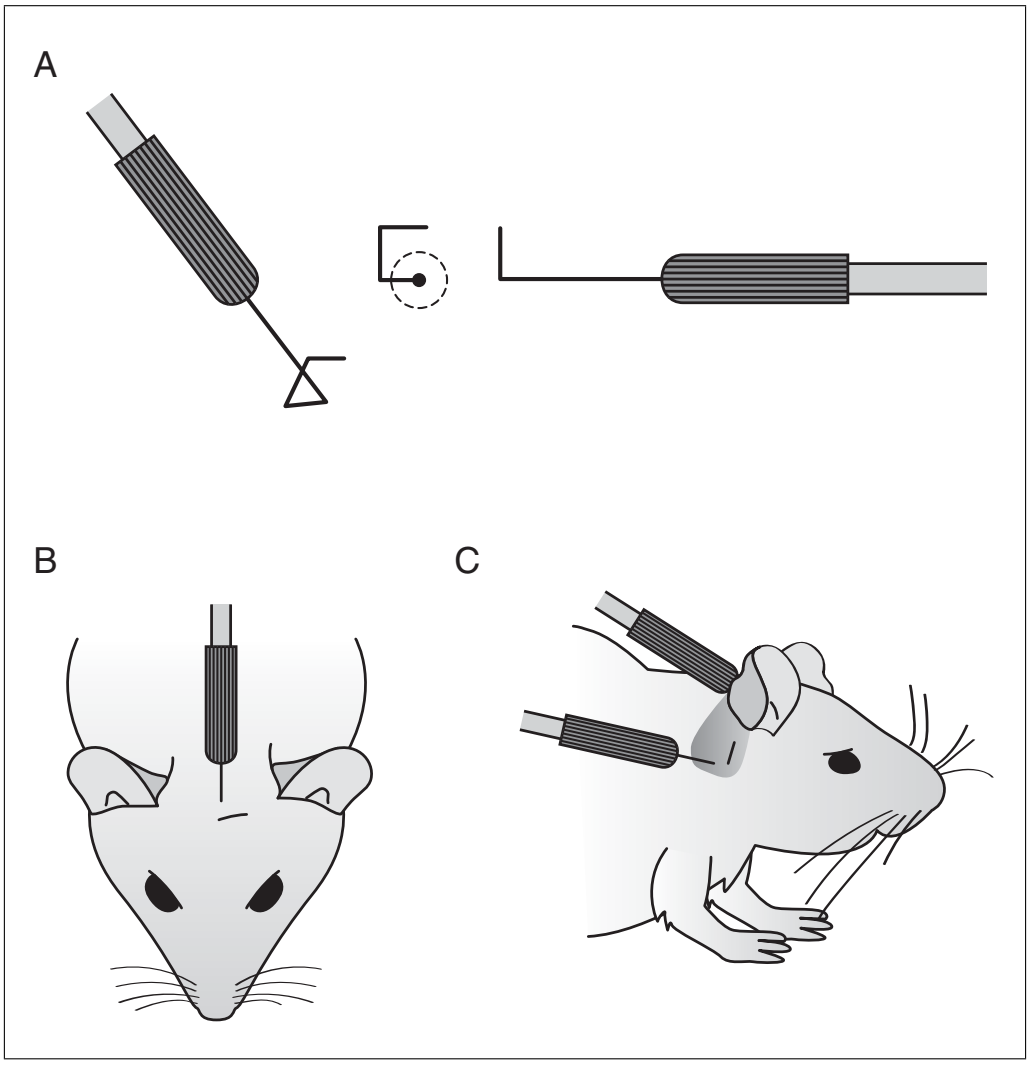

Figure 1 Positioning of sub-dermal electrodes for ABR recording. (**A**) Electrode configuration, to demonstrate the hook introduced onto commercially available EEG electrodes. The three views, from left to right, indicate an oblique view, an end-on view and a side view of the hook in the needle electrode. (**B**) The positioning of the active electrode on the vertex of the mouse. (**C**) The positioning of the reference and ground electrodes in the patch of bare skin behind the pinna overlying the bulla.

3. Once sufficient depth of anesthesia is achieved, as determined by abolition of the righting reflex and pedal withdrawal reflex, and following calibration of the sound system (step 2, above), insert needle electrodes (active electrode on the vertex, reference electrode overlying the left bulla, ground electrode overlying the right bulla; Fig. 1). Lay the mouse in a natural position, facing the loudspeaker, at a distance of 20 cm from the loudspeaker leading edge to the mouse interaural axis (Fig. 2).

Record ABRs

4. Set up the sound system equipment (Fig. 2) and calibrate the sound delivery system each day. Position the loudspeaker oriented along the mouse's interaural axis 20 cm in front of where the mouse will subsequently be placed. Position the microphone (ACO 7017) where the mouse's ears will be. Present 500-msec noise bursts 10 times.

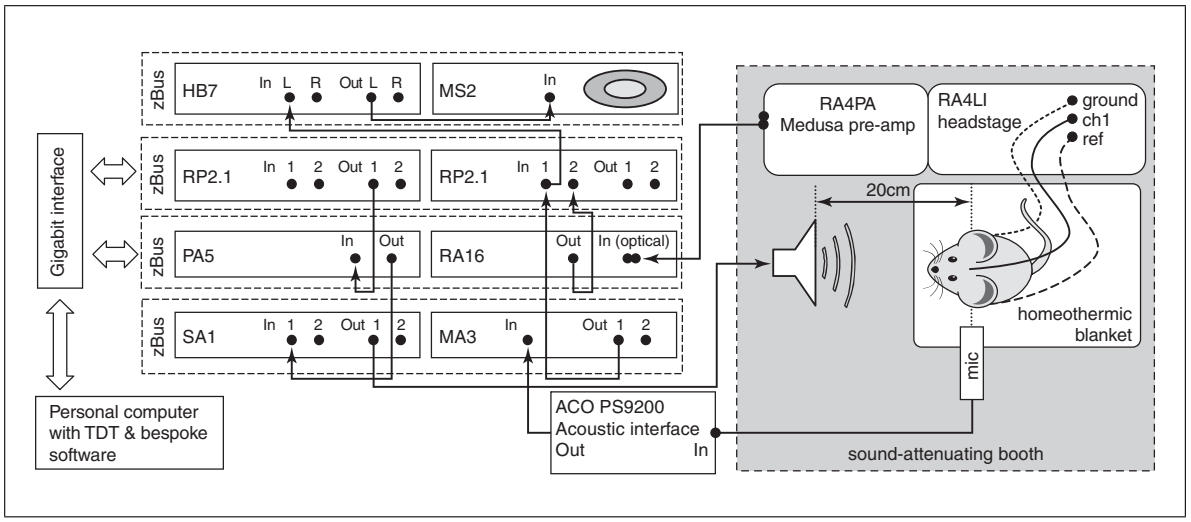

Figure 2 A schematic to illustrate the ABR setup used for high-throughput screening with free-field sound stimulation. The mouse is shown, with electrodes attached, on a heating blanket, with 20 cm between the leading edge of the loudspeaker and the mouse's interaural axis. For sound system calibration, the microphone is positioned where the center of the animal's head will be later positioned, oriented along the line of the interaural axis. Each TDT System3 module is housed within a zBus caddy. A gigabit (or optibit) interface provides communication between programmable modules and the computer system.

Using the MA3 amplifier at 40 dB gain, amplify microphone signals (from the ACO 7017) and digitize at 100-kHz sample rate (RP2.1). Subject each response to a Fast Fourier Transformation (FFT) and analyze the average FFT to produce an equalization curve, which is then used to ensure that all ABR stimuli are presented at a known sound pressure level (accounting for the frequency response of the sound transducer). Digitally generate stimuli under software control and convert to an analog waveform in the RP2.1 processor at 100-kHz sampling rate at 5 V pk-pk amplitude. Use the PA5 Programmable Attenuator to attenuate the waveform to produce the desired sound pressure level according to the calibration/equalization curve.

By generating a large amplitude waveform in the RP2.1 digital-analog converters (DACs) followed by attenuation, an improved signal-to-noise ratio for the stimulus is produced; any noise inherent to the DAC is attenuated along with the signal. This is of particular importance for the production of the very low sound pressure level stimuli, critical for assessment of auditory sensitivity. If a lower amplitude signal waveform is generated on the DAC, removing the need to use the analog attenuators, then DAC noise is higher relative to the signal amplitude and a poorer signal-to-noise ratio results, which can result in noise masking of very low amplitude signals and a poorer estimate of pure tone ABR thresholds. The attenuated signal is then amplified and sent to the loudspeaker, producing the required sound pressure level at 20-cm distance from the loudspeaker, where the mouse's head is now positioned.

5. Record a test ABR trace to ensure the system is functioning correctly, using clicks at 70 dB SPL (sound pressure level).

6. Record a series of ABRs using an array of click stimuli (a 10-μsec duration positive transient, 42.6/sec, 256 sweeps at a fixed phase), from 0 to 85 dB SPL in 5-dB steps.

   These responses are used to determine the ABR threshold to the click stimuli.

7. Record a series of ABRs using an array of stimuli of various frequencies and SPLs.

   The run presents tones pips (5 msec duration, 1 msec rise/fall time, 42.6/sec, 256 sweeps at a fixed phase) across five frequencies (30, 24, 18, 12, and 6 kHz) at intensity levels

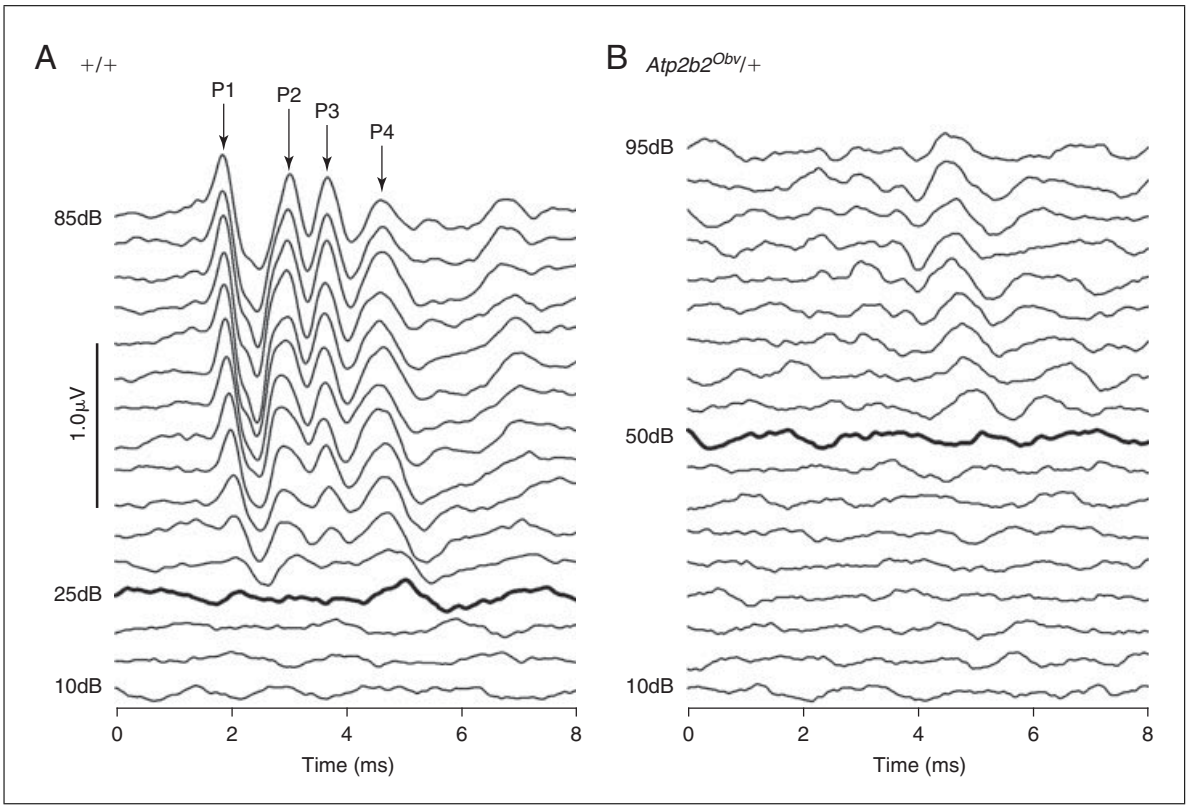

Figure 3 Click-evoked ABR traces recorded from a control and *Atp2b2*^{Obv}/+ mouse. ABRs were recorded in response to clicks at 5-dB incremental sound levels from 10 to 85 dB SPL in wild-type mice (**A**) and from 10 to 95 dB SPL in mutant mice (**B**). The estimated threshold ABR is plotted as a heavy trace and the threshold indicated adjacent. Four positive peaks are clearly indentified in control mice (indicated by P1, P2, P3, and P4). For this mutant, the ABR waveform was significantly altered (P1-P3 were not present), as well as having a high threshold.

increasing from 0 to 85 dB SPL in 5-dB steps. For routine screening, different SPL ranges are recorded for different test frequencies to improve time efficiency (6 kHz, 20 to 85 dB; 12 kHz, 0 to 70 dB; 18 kHz, 0 to 70 dB; 24 kHz, 10 to 70 dB; 30 kHz, 20 to 85 dB). Responses are recorded at each stimulus level, in decreasing frequency order before stepping up to the next highest stimulus level. These responses are used to determine the ABR thresholds to the tone stimuli.

If mice appear to have hearing impairment, the upper limit of SPLs is extended to 95 dB for each test frequency and for clicks (this represents the upper limit of the linear range of our sound system at these frequencies).

8. Record a final test trace, again using clicks at 70 dB and compare with the initial test trace to ensure there has been no deterioration in the response during the measurements.

Promote recovery

9. Promote recovery of the mice from anesthesia with a 10 ml/kg intraperitoneal injection of atipamezole mix (0.1 ml/10 g body weight containing 0.01 mg atipamezole/10 g body weight). Return the mouse to its cage, place on a thermal mat or in a temperature-controlled ventilated rack. Monitor the recovering mice over the next 1 to 3 hr and return them to the holding racks once they are able to move well and respond to external stimuli.

Analyze the data

10. Analyze data files to visualize the ABR waveforms and allocate thresholds. Organize ABR traces such that increasing dB SPL responses for a particular test stimulus are stacked together, aligned on the abscissa (time, msec; see Fig. 3)

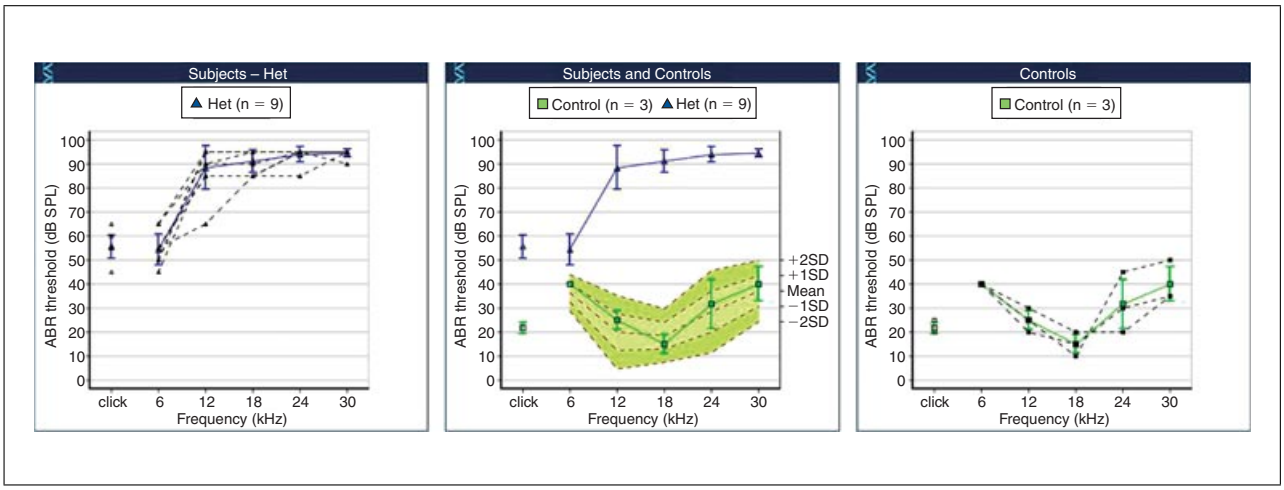

Figure 4 ABR audiograms for wild-type and *Atp2b2*^{Obv}/+ mice. ABR thresholds are plotted for individual wild-type and mutant mice in the right and left panels, respectively. Mean ABR thresholds (± standard deviation, SD) are plotted for wild-type (green) and *Atp2b2*^{Obv}/+ mice (blue) for the range of frequencies tested and for clicks. In the center panel, mean thresholds (± SD) are re-plotted for wild-type (green) and *Atp2b2*^{Obv}/+ mice (blue) over a reference mean threshold (±1 SD and 2 SDs and shown as lighter green and darker green areas, respectively) calculated from a large population of wild-type mice on the same genetic background.

11. For each stimulus, determine the threshold by visual inspection of the trace stack as the lowest stimulus level (dB SPL) where any recognizable feature of the waveform can be identified (Fig. 3).

    As the stimulus level decreases, waveform amplitude reduces and wave peak latency extends. These trends are useful to help to identify true waveform features for determination of threshold.

12. For different cohorts of mice, calculate mean thresholds for each test stimulus and plot as a function of the test frequency, in addition to plotting the individual mouse response thresholds. Click thresholds are plotted at an arbitrary labeled point on the frequency abscissa to separate them from pure tone thresholds, which are plotted linked by a line (see Fig. 4). Plot control and mutant thresholds together to facilitate comparison of mean thresholds and add a baseline, reflecting the mean threshold (± 1 and 2 standard deviations) of a large population of control mice on the same genetic background.

    If the mutant mean threshold for any test stimulus exceeds ±2 SDs from the baseline average, the mutant line is considered to exhibit a hearing impairment phenotype (see Fig. 4).

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps.

Atipamezole mix

Mix 0.2 ml antisedan (Atipamezole hydrochloride 5 mg/ml; Pfizer) and 9.8 ml H_2O (Atipamezole 0.1 mg/ml)

Ketamine/xylazine anesthetic mix

Mix 1 ml ketamine (Ketaset 100 mg/ml; Fort Dodge Animal Health), 0.5 ml xylazine (20 mg/ml; Rompun 2%, Bayer), and 8.5 ml H_2O.

continued

Store the anesthetic mix and recovery agent dilutions for a maximum of 7 days before it is disposed of via incineration (after binding with Clan Uni-Safe chemical spillage reagent; Fisher Scientific) and keep refrigerated at 4°C when not in use. Solutions should be warmed to room temperature before use. Stock bottles are stored at room temperature and any unused contents disposed of after 28 days using authorized local protocols.

COMMENTARY

Background Information

The ABR method has been widely used in clinical and research settings for many years. It provides an accurate means to estimate hearing sensitivity using electroencephalographic measures rather than behavioral responses. Because the recordings are derived from brainstem activity, with little or no contribution from higher centers, the measurements are largely unaffected by the use of anesthetic agents.

Other methodologies can be used as screening methods for hearing with recovery of the mouse after the testing procedure. Testing for the Preyer reflex gives a crude indication of the presence or absence of hearing at high stimulus intensity but it will not detect mild or moderate hearing impairments. Otoacoustic emissions (distortion product, DPOAEs) can also be employed as a rapid screening method (Martin et al., 2006), but these measurements will only detect outer hair cell or middle ear pathology and will not identify as wide a range of hearing deficits as ABRs. The auditory steady-state response (ASSR) can also potentially be used as a screening technique based on electroencephalographic recordings similar to ABR (Pauli-Magnus et al., 2007).

The ABR technique presented here is an optimized, fast recording protocol (approximately 15 to 20 min per mouse). It yields high-quality recordings and reliable estimates of thresholds in mice anesthetized with an easily reversible agent. This optimized protocol can be easily integrated into high-throughput phenotyping pipelines, which can cover a wide range of biological screening tests. Using a combination of click-evoked and tone-evoked ABRs, it is possible to identify more potential deafness-associated mutations than with the Preyer reflex testing or click ABR screening alone. Observations of Preyer reflex alone will not detect mutants showing mild or moderate hearing impairment. By using tone-evoked ABRs, it is possible to identify mutants with hearing deficits in particular frequency domains (e.g., mice showing severe high frequency deficits can have normal low frequency and click-evoked ABR thresholds).

Critical Parameters and Troubleshooting

Anesthesia

1. Use artificial tears (Viscotears) during the procedure to prevent corneal drying, thus improving the welfare of the mice in recovery after the recording procedure.

2. Use a heating pad/blanket to maintain body temperature in the mice. Anesthetized mice will become hypothermic very quickly and the accompanying drop in metabolic rate will seriously affect the efficiency of inner ear function and result in artifactually high thresholds.

3. Ensure that the mouse achieves a sufficient depth of anesthesia. With the indicated dose of ketamine/xylazine, mice will quickly lose their righting and corneal reflexes. Abolition of pedal withdrawal reflexes may take a few minutes longer. It is important to achieve this depth so that the animal does not feel the insertion of the needle electrodes. Furthermore, if the mouse remains only lightly anesthetized, it will maintain a higher degree of muscle tone, which will introduce excessive myogenic electrical activity on the recorded traces. It is important to minimize sources of electrical noise arising from insufficient anesthesia to prevent the low-amplitude ABR waveform from being masked. Such masking can produce an artificial elevation of the estimated ABR threshold as the peak and trough features of the waveform used to identify an ABR will become buried in the noisefloor of the recording and only become visible at higher dB SPLs.

Equipment

1. Use a sound-attenuating booth to reduce background (masking) acoustic noise. The booth should also be grounded (and thus act as a Faraday cage) to ensure low levels of ambient electrical noise, which can interfere with low-impedance recording systems.

2. Ensure that the needle electrodes are inserted sub-dermally. This provides better electrical contact with the animal and reduces electrical noise on the recorded traces.

3. The microphone used for daily calibration of the recording system should itself be regularly calibrated (e.g., quarterly or biannually) to ensure that the sound levels presented to mice are consistent over long periods of time.

Recording

1. The stimulus and recording parameters described in this protocol provide a reliable and time-efficient method to estimate ABR thresholds in a high-throughput phenotyping screen. The relatively low number of sweep records (256) contributing to each averaged waveform is made possible by minimizing sources of acoustic and electrical noise in the recording environment. In addition, the stimuli are presented relatively rapidly (42.6/sec), which helps to desynchronize any potential noise from the 50-Hz mains electricity supply. Many other studies use a slower rate of presentation (<30/sec). However, the higher stimulus presentation rate has a minimal effect on the recordings and facilitates a shorter time period before the mouse can be allowed to recover from the anesthesia, thus helping to improve the welfare of the mice.

2. As no artifact rejection is used in the recording system it is possible that the averaged trace can be significantly influenced by ECG activity, which happens to synchronize to the stimulus repetition rate. This is a rare occurrence and, if necessary, the affected stimulus frequency/level combinations can be repeated manually once the main automated data collection is complete.

Anticipated Results

Examples of ABR audiometric profiles recorded in control (wild-type) and a hearing-impaired mutant mouse are shown in Figure 4. Using the recording protocol detailed here, mice tend to have the highest sensitivity of hearing (lowest thresholds) at 12 to 18 kHz, with average thresholds being around 10 to 20 dB higher at 6 kHz and 30 kHz. Click threshold often equates well with highest sensitivity tone threshold.

Examples of ABR thresholds in hearing-impaired mutant mice are shown in Figure 4. Average thresholds for the *Atp2b2*^{Obv} heterozygotes are significantly elevated compared to those of control mice on the same genetic background (Spiden et al., 2008). The use of ABR recordings to screen for hearing impairment is more informative and they will identify more lines of interest than screens using only a test for the presence or absence of a Preyer reflex. Using the ABR method, the sensitivity of hearing across a range of frequencies can be estimated. This allows for even mild-to-moderate degrees of hearing impairment to be detected. The use of a range of test frequencies also gives a more comprehensive description of any hearing impairment detected. The use of click-evoked ABRs alone will give a good estimate of overall hearing sensitivity, but will not detect frequency-specific deficits in hearing; for example, loss of high frequency sensitivity would be missed as such lines can show normal sensitivity to low frequency tones and broadband clicks.

Time Considerations

Using this protocol, it is possible to overlap the experiment on subsequent mice; for example, while one mouse is in the recording booth, the next mouse can be given its anesthesia injection. Induction of anesthesia requires ~6 to 8 min following injection and prior to the start of electrophysiological recordings. Once in place in the booth, all ABRs can be recorded in 10 min using the semi-automated software routines.

A minimum of four mutant mice per line should be subjected to ABR recording. More mice are subsequently tested if initial results show features of interest. Additionally, four to eight wild-type mice per week (from the same genetic background as the mutants) should be subjected to ABR recording. At full pipeline capacity, 44 to 48 mice per week can be tested and fully analyzed (plus any additional mice exhibiting ABR behavior warranting further examination).

Allowing ~15 min per mouse (overlapping anesthetic inductions and recordings), four mice can be tested per hour. This equates to ~12 to 13 hr of experimental work per week, plus ~1 hr set up/clean-up time per experimental day. Allowing for ~5 min per mouse to upload data and allocate thresholds totals 3 to 4 hr of analysis per week. General organization and scheduling requires ~3 to 4 hr per week. An additional 3 to 4 hr per week is required for quality control of analyses and project management.

Thus, approximately 30 working hours per week are required to screen the hearing of 44 to 48 mice. This allows time for testing of additional mice from lines already identified as being of interest.

Acknowledgments

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Internet Resources

http://www.sanger.ac.uk/mouseportal

Web site for phenotyping and mutant mouse resources at the Wellcome Trust Sanger Institute.