Assessment of Cochlear Function in Mice: Distortion-Product Otoacoustic Emissions

The term otoacoustic emissions (OAEs) refers to sounds that can be measured in the ear canal following the presentation of an acoustic stimulus. These sounds are generated by the sensory cells of the inner ear, namely, the outer hair cells (OHCs). What makes OAEs useful for the assessment of auditory function is that they are reduced or absent when the OHCs are damaged by, for example, exposure to excessive noise or ototoxic drugs. Consequently, OAEs provide a powerful and noninvasive means to assess the robustness of OHC function.

OHCs have the unique property of being motile so that in response to an acoustic stimulus, this motility amplifies the vibrations of the basilar membrane, thus accounting for a large part of both human and animal auditory sensitivity to sound. In the process of amplification, reverse traveling waves are generated that vibrate the middle-ear ossicles and tympanic membrane (much like a loudspeaker), thereby producing an acoustic signal in the ear canal. OAEs can be evoked by presenting a variety of acoustic stimuli to the ear canal, with the most common being clicks, single pure tones, or tone pairs.

When two tones are presented to the ear canal, a distortion-product otoacoustic emission (DPOAE) is produced. This emission arises because of the nonlinear aspects of OHC transduction in which new frequencies are generated that are not present in the input signal. Although families of DPOAEs are evoked by a two-tone stimulus, the largest and most prominent emission occurs at the $2f_1$-f_2 frequency, where f_1 is the lower-frequency tone and f_2 is the higher-frequency tone. The advantage of DPOAEs is that tone pairs can be selected to test practically any frequency of interest within a species' hearing range. Additionally, these emissions are large and robust in laboratory animals. These factors make DPOAE measurement particularly attractive for assessing auditory function in laboratory animals such as mice.

Typically, tone pairs, often referred to as primaries, are presented to produce DPOAEs with a frequency resolution of five to ten points per octave and the level of the resulting emission is measured for each primary-tone pair. An octave is equivalent to a doubling of frequency and because frequency mapping is scaled logarithmically along the cochlea, octaves roughly represent equal cochlear distances. Thus, the $2f_1$-f_2 DPOAE is measured five to ten times from 1 to 2 kHz, 2 to 4 kHz, and so on, over the frequency range of interest. The resulting DPOAE levels are plotted as a function of frequency to produce what is referred to as a DP-gram because of its many similarities to a clinical audiogram. The clinical procedure actually measures hearing by asking a subject to respond if they can detect a tone, and the level of the softest tone they can hear is plotted as a function of test frequency. This unit presents methods to measure DPOAEs in mice. It is important to note, however, that DPOAEs have also been measured in rabbits, rats, gerbils, guinea pigs, chinchillas, nonhuman primates, and a variety of less common laboratory species such as bats, frogs, and lizards. Thus, these methods are applicable to essentially all small laboratory animals.

COMPUTER CONTROL AND DPOAE MEASUREMENT

BASIC PROTOCOL

A personal computer is used to either control the stand-alone equipment or simulate these functions in software via a commercially available soundcard. For evoking DPOAEs, the primaries are presented to the ear canal while the ear-canal signal picked up by the

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microphone is simultaneously analyzed either by a dedicated spectrum analyzer or by fast Fourier transform (FFT) software programmed for the soundcard. The two tones, f_1 and f_2, are presented with a known relationship called the f_2/f_1 ratio that is typically near 1.25 for mice. Because DPOAEs are very low-level sounds, the ear-canal signal from numerous readings must be summed and then averaged to determine the DPOAE. Typically, with a stand-alone spectrum analyzer, individual spectra are computed and then averaged. In other words, the amplitude of the signal in each frequency bin of the spectrum is computed and corresponding bins in subsequent spectra are averaged. This technique, called spectral averaging, does not actually reduce the noise floor (NF) of the measurement system. Because DPOAEs are quite large in mice and other rodents, this method is adequate for most purposes. For better noise reduction, the ear-canal signal can be averaged as a time waveform so that unwanted noise cancels over time. Then, the spectrum of this averaged time waveform is computed after the final sample. This technique, known as time or synchronous averaging, reduces the NF in a manner identical to that used to measure, for example, evoked neural signals or potentials from the brain. To efficiently implement this procedure for DPOAEs, the two tones (f_1 and f_2) must be adjusted so that an even number of cycles of each tone fits the sample window used to acquire the ear-canal signal. In other words, primary tones must begin and end on the same part of the sine wave cycle, for example, zero crossing. Because the sampling frequencies are hardwired into many boards and the number of points sampled is fixed by the size of the FFT, the actual frequencies of the primary tones need to be slightly adjusted to make each tone exactly fit the sample window. Since the DPOAE derives from the formula $2f_1 - f_2$, this procedure also makes the DPOAE exactly fit the sample window so that the emission is averaged while random noise is canceled.

In reality, spectra are based upon digitized samples of the ear-canal signal that is to undergo spectral analysis. These points are collected at some sampling frequency usually for a number of points based upon a power of two. The authors typically sample at 44,100 Hz, a common sampling frequency used by soundcards, and collect 4096 points. Computation of the spectra results in 2048 bins, each with a frequency resolution of 10 Hz from 0 to 22 kHz. The DPOAE of interest occurs at the $2f_1 - f_2$ frequency and, by appropriate calculations, the bin of the spectrum that corresponds to this frequency can be determined. Because DPOAEs simply rise above the noise of the measurement system, it is necessary to not only measure the level of the signal corresponding to the DPOAE frequency, but also some index of the NF of the measurement system must be obtained. This can be accomplished by measuring the level of the ambient noise present in several frequency bins that are 50 to 100 Hz below the DPOAE frequency, and determining the average and standard deviation (SD) of these bins. A DPOAE is often considered present when it is at least 3 dB above the average NF. A more conservative measure is to require that the DPOAE be >2 SDs above the NF. Figure 8.21C.1 shows an actual spectrum of the ear-canal signal of a mouse in which the $2f_1 - f_2$ DPOAE, NF, and primary tones are indicated along with several other prominent DPOAEs measurable in rodent ears.

The final aspect of computer control is to generate primary tones that increment in frequency so that the f_2 frequencies are presented between five and ten points per octave. Both the level of the DPOAE and its corresponding NF are saved to disk as dB sound pressure levels (SPL). These two values are usually plotted as a function of f_2, or as the geometric mean of the two tones as the test proceeds, so the experimenter can monitor the results. The resulting DP-grams can be collected at a number of levels of the two primaries to generate measures with varying sensitivity to cochlear damage.

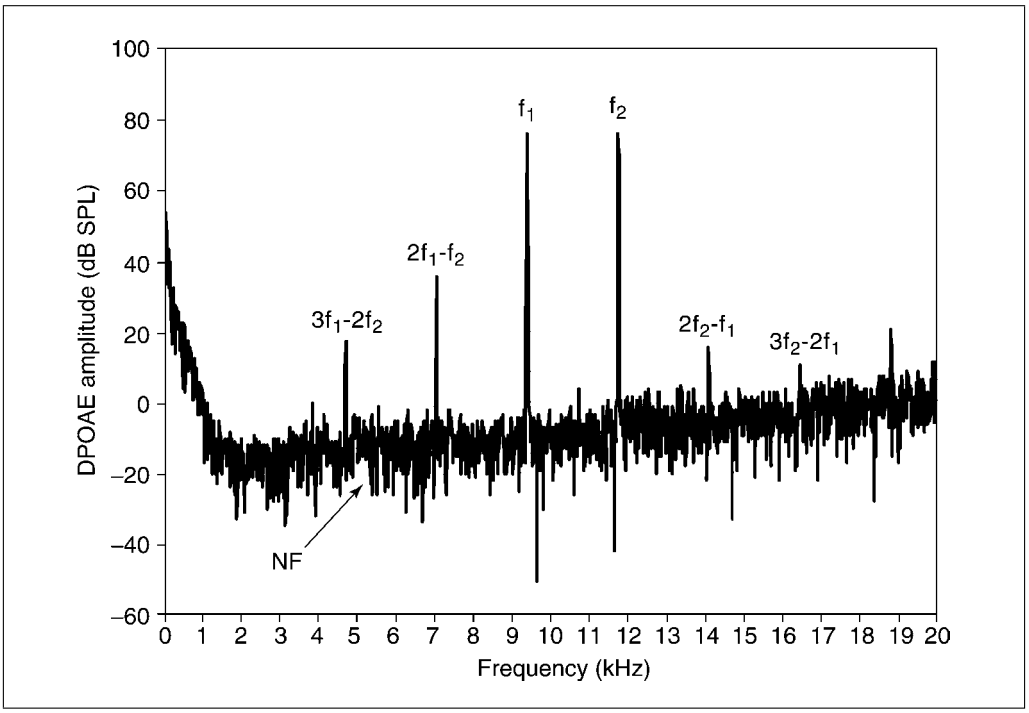

Figure 8.21C.1 Spectrum of the ear-canal signal from a mouse showing primary tones f_1 and f_2 and the several upper- (e.g., $2f_2$-f_1) and lower-sideband (e.g., $3f_1$-$2f_2$) DPOAEs commonly observed in rodent ears. It is clear that the $2f_1$-f_2 DPOAE is the largest emission and for this reason it is the DPOAE most commonly studied. The NF is indicated by the arrow and, in this example, the NF is approximately −10 dB SPL.

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

Anesthesia (e.g., ketamine/xylazine, Avertin, or a gaseous anesthetic such as isoflurane; *APPENDIX 4B*)

Two channel, low-distortion frequency synthesizer (e.g., 33220A Function Generator, Agilent Technologies) or two-channel computer soundcard (100 Hz to 60 kHz frequency range; e.g., LynxTWO, Lynx Studio Technology)

High-frequency tweeters to produce pure tones from 100 Hz to at least 60 kHz (e.g., ES1/EC1 electrostatic speakers, Tucker-Davis Technologies)

Low-distortion amplifier for impedance-matching speakers to sound sources (e.g., HB-7 headphone buffer, Tucker-Davis Technologies)

Programmable attenuator to adjust the levels of the two tones (e.g., PA5 programmable attenuator, Tucker-Davis Technologies)

Specialized low-noise microphone/amplifier for measuring ear-canal sound pressure (e.g., ER-10B$^+$ emissions microphone, Etymotic Research) fitted with sound-delivery tubes supplied by the vendor for delivering acoustic stimuli and an internal port for microphone pickup (DPOAE frequency range = 1 Hz to 35 kHz)

Sound-delivery tubes to connect high-frequency tweeters to microphone assembly (2-mm i.d.)

Sound-attenuation chamber: commercially available (Acoustic Systems or Industrial Acoustics Corporation) or laboratory-made sound-attenuation enclosure (see step 3)

Temperature-controlled heating pad or heating table (e.g., Harvard Apparatus)

Spectrum analyzer (e.g., 35670A FFT dynamic signal analyzer, Agilent Technologies) or computer soundcard (e.g., LynxTWO, Lynx Studio Technology) with spectral analysis software (range = 1 Hz to at least 50 kHz)

1/4-in. high-frequency microphone (e.g., type 4136 pressure microphone, Bruel & Kjaer) and associated preamplifier (range = 1 Hz to 100 kHz)

Sound calibrator (QC-20 sound calibrator, Quest Technologies)

1-ml syringes

Teflon tape

Personal computer equipped with an instrument controller card (e.g., NI-PC/104-GPIB, National Instruments) to control the frequency synthesizers and spectrum analyzer for a stand-alone setup or a soundcard with appropriate software to substitute for these instruments

Small pieces of silicon tubing to use as probe tips for the microphone assembly (ER3-34 infant silicon tips, Etymotic Research)

Small, curved forceps

Set up equipment for stimulus generation and presentation

1. Connect the output of the sound-generating equipment (frequency synthesizers/soundcard) to the high-frequency tweeters through a low-distortion amplifier to match impedance between the two devices.

2. To control stimulus levels, connect programmable attenuators before the connection to the tweeters.

   For computer-generated stimuli, attenuation can be performed programmatically, but attenuation in excess of 70 dB results in stimulus degradation.

3. Connect the conical tweeter housing that tapers to a small orifice to the sound-delivery ports of the microphone with small-diameter tubing. Place the tweeters and microphone assembly within a sound-attenuation chamber (see Fig. 8.21C.2).

   A laboratory-made sound-attenuation chamber is highly desirable (to eliminate background noise that makes DPOAE recording difficult or impossible, depending upon the ambient noise levels). The chamber can be a wooden, double-walled enclosure with ~2 in. of styrofoam-type insulation between the walls. Ideally, the box should include a small double-pane window for observing the mouse during testing and should be large enough to accommodate the heating pad. The chamber should have a shelf as a support structure to hold the microphone/speaker assembly. An iron plate with adjustable magnets and clamps is also very helpful to hold the speaker assembly. A self-retaining retractor (e.g., self-retaining Codman surgical retractor) to hold the probe can also be useful. Most environmental noise, such as air-conditioning fans or other noise-producing equipment, is <4 kHz. Because mouse DPOAEs are >4 to 6 kHz, sound isolation is less important for mouse work. One possibility would be to modify a large ice chest as a test enclosure. One of the most important considerations is designing the box so that one can easily reach the pinna for placement of the probe.

4. Connect the output of the microphone/preamplifier to the input of the spectrum analyzer or soundcard.

Sound calibration

Sound calibration is a difficult problem because of standing waves that occur in cavities (e.g., outer-ear canals) of various lengths and dimensions. There are many opinions regarding how calibrations should be performed. Pearce and co-workers (2001) discuss many of these issues, especially with respect to the mouse. The goal is to present the desired SPL at the tympanic membrane and measure the correct DPOAE level in the ear canal. The most simple method involves simulating the ear canal by a small cavity of similar volume (steps 5 to 7 below). This cavity can be a 1-ml syringe with the needle end cut off and adjusted to 0.1 ml, i.e., the approximate volume of the

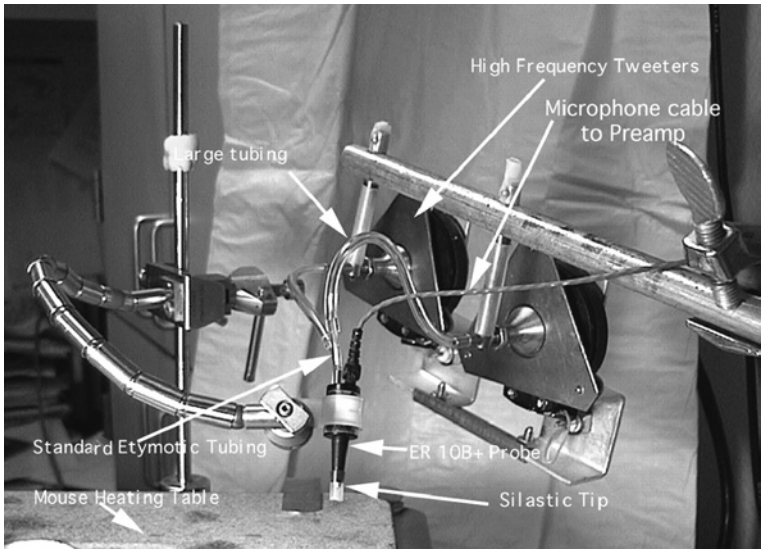

Figure 8.21C.2 Photograph of the setup for testing mouse DPOAEs. High-frequency tweeters are shown at right encased in conical housings so that small polyethylene tubes can be used to couple them to the emissions probe shown in the center foreground. Speaker sound tubes couple with tubing supplied with the emissions microphone (Etymotic Research). The small tubing connects directly to two sound ports that pass through the microphone. Preamplifier cable from the microphone can be seen leaving the probe at the right. The probe is fitted with small silicon tubing (ER3-34 infant silicon tips, Etymotic Research) to help seal the probe into the mouse ear canal. In this photograph, a self-retaining Codman surgical retractor at left holds the probe. However, the tension of speaker tubing can also hold the probe in place. When the probe is properly placed in the ear canal, the fit is often secure enough to be able to lift the head of the mouse off the heating table by pulling up on the probe.

mouse ear canal. Calibration in a small cavity has the advantage of keeping the protocol relatively simple and constant and does not incorrectly adjust the level of the primaries when standing waves are encountered at different frequencies in individual ears (see Whitehead et al., 1995b). However, this method does not permit customized adjustments for the idiosyncrasies of individual ears. Calibration of the system output and emissions probe microphone is performed once and then periodically checked. These values are changed only if subsequent calibrations reveal significant discrepancies from the original calibration procedure. The alternative method attempts to customize the output for each ear by producing an in-the-ear correction for each mouse (step 8).

Calibrate sound output in a small cavity

5a. Produce a 1-kHz signal of known SPL by placing the 1/4-in. microphone in the sound calibrator and feed the output of microphone preamplifier to the spectrum-analysis equipment.

A sound calibrator is a commercially available device that produces a 1-kHz tone usually at 95 dB SPL.

6a. Collect a spectrum of the microphone signal that reveals the 1-kHz peak and set the scale of the analyzer screen to read in dB SPL based upon this peak.

Because the 1/4-in. microphone response is designed to be relatively flat as a function of frequency, other frequencies can be calibrated based upon this calibration at 1 kHz.

7a. Seal the 1/4-in. microphone in one end of a cut-off 1-ml syringe cavity using Teflon tape wrapped around the microphone to produce a tight fit and seat the DPOAE probe in the other end of the cavity cut so that the volume between the probes is ~0.1 ml

(similar to Fig. 8.21C.4). Either manually or under computer control, present the various primary tones unattenuated to cover the range of mouse hearing to be tested and measure the maximum output of the system. Proceed to step 8.

This provides a calibration table so that primaries of known SPL can be presented by applying the appropriate attenuation.

Calibrate sound output in the ear

5b. Alternatively, use the emissions microphone to calibrate the primary-tone levels in every ear before the test begins. Proceed to step 8.

This method results in slightly different voltages to the speakers for each animal based upon probe position with the assumption that this adjustment keeps the SPL at the tympanic membrane constant across animals. However, when a cancellation at the probe microphone is encountered due to standing waves, the measured primary tones will appear to be too low in level and the computer will, consequently, incorrectly increase the primary-tone outputs. Similarly, when standing waves reinforce one another, the measured primaries will appear to be too high in level and errors in the opposite direction will occur. The authors prefer the cavity technique (steps 5a to 7a), because all parameters are kept constant across animals and, if a problem develops, it is often easier to either determine the element that is not functioning correctly or make some sort of correction after the fact. However, when the sound is adjusted for every animal, it is much more difficult to make any corrections, for example, if an attenuator was not set to the correct value. Others would argue that it is more important to try to present the stimuli as accurately as possible to individual ears. For a novice, it is recommended that the cavity method be used until one becomes thoroughly familiar with measuring DPOAEs.

Calibrate emissions probe microphone

The DPOAE microphone can be calibrated to compensate for loss of sensitivity at high frequencies. In this case, tones of known SPL across the usable sensitivity range of the microphone (0 to 35 kHz for the recommended Etymotic ER-10B$^+$ microphone) are presented to the DPOAE probe that houses the actual emissions-measurement microphone. This procedure is also performed in a cavity similar in size to the mouse ear canal (step 7a), but sound is introduced from one end via one of the mouse tweeters and the DPOAE probe is inserted into the other end opposite the speaker input.

8. Step the tones throughout the sensitivity range of the DPOAE microphone with the 1/4-in. microphone in place of the DPOAE probe and determine the output of the tweeter as a function of frequency.

Based upon these measurements, it is possible to adjust the output of the tweeter to be flat across frequency. For example, the output of the tweeter can be adjusted so that the sound pressure in the cavity is 60 dB SPL for every frequency.

9. Place the DPOAE probe in place of the 1/4-in. microphone and measure the output of the DPOAE microphone as a function of frequency.

10. Record deviations from the flat response of the sound source to use as correction factors that are applied to the probe microphone for a dB SPL reading.

These calibration procedures result in tables that are used by the computer to output the requested primaries in dB SPL and to adjust the DPOAE level measured by the emissions microphone to correctly read in dB SPL (e.g., see Table 8.21C.1).

11. To determine the probe fit in the ear canal, use the DPOAE program, for example, by presenting 75-dB SPL tones at 1 and 2 kHz and display the corresponding spectrum of the ear-canal signal with a reference line to indicate the 75-dB SPL level. If the tones do not meet the desired levels, reposition the probe and repeat the “check fit.”

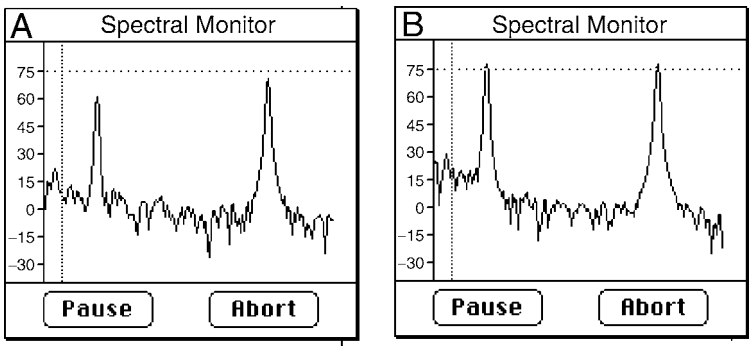

Figure 8.21C.3 Example of a "check-fit" window used for judging the quality of the placement of the probe in the ear canal. (**A**) A poor check-fit in that both primary tones are too low and do not converge on the 75-dB SPL reference line (dotted line) near the top of the window. Repositioning the probe (**B**) results in a good check-fit with both primaries extending to the reference line.

Figure 8.21C.3 illustrating a "check fit" measurement from a mouse using the authors' DPOAE program shows a poor fit in which both primary tones are too low in level (A) and a good fit (B) once the probe was repositioned. Although very simple, this method seems to greatly improve the reliability of the test.

Collect DP-grams

12. Place the emissions probe in a 1-ml syringe with the needle-end cut off and the plunger adjusted to 0.1 ml to approximate the volume of the mouse ear canal (see Fig. 8.21C.4). Perform a test run in the cavity to verify that the equipment is operating correctly.

    This test is especially useful to be certain no unwanted distortion is present or that the NFs are not abnormally high.

    Because poor electrical connections affect these measurements, it is always advisable to ensure that the equipment is functioning properly before anesthetizing a mouse.

13. Turn on the heating pad and allow it to reach the mouse's body temperature of 37°C.

    It is extremely important to maintain body temperature at this level or DPOAEs will be adversely affected. Because of the small size of the mouse, most rectal probes cannot be inserted and should instead be placed so that they make firm contact with the underside of the body. A small gooseneck lamp positioned near the mouse can provide additional warmth. However, make sure the lamp is not too close because it can overheat the animal.

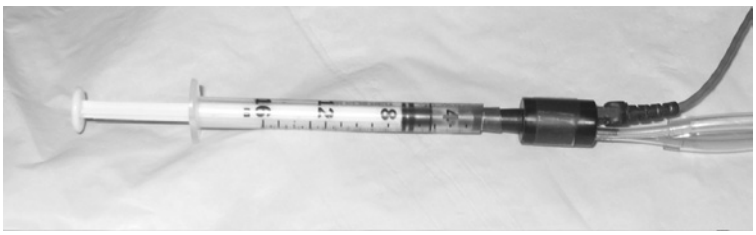

Figure 8.21C.4 Photograph of a 1-ml syringe with the needle-end cut off, which is used as a standardized test cavity. The DPOAE microphone with silicon tip is positioned in the cutoff end and the plunger is adjusted to ~0.1 ml to approximate the volume of the mouse ear canal.

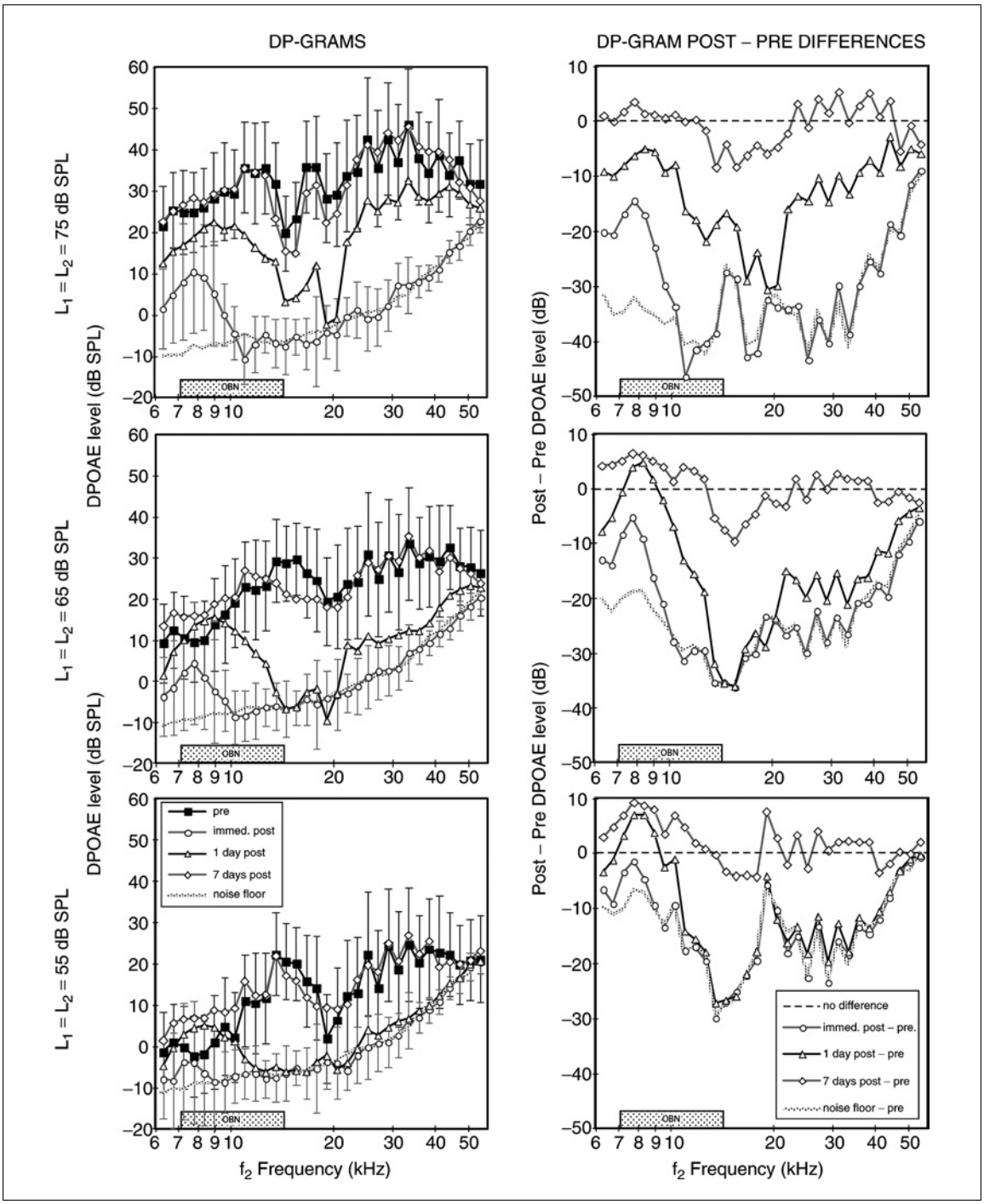

Figure 8.21C.5 Legend at right.

14. Fit the probe microphone with a small piece of silicon tubing prior to insertion into the ear canal.

    This tubing helps to protect the tympanic membrane, but more importantly it serves to acoustically seal the probe into the ear canal.

15. Prior to placing the probe in its ear, anesthetize the mouse using, e.g., a mixture of ketamine/xylazine, Avertin, or a gaseous anesthetic such as isoflurane (*APPENDIX 4B*).

    Once the appropriate anesthetic has been chosen in consultation with the veterinarian in charge, the investigator must receive hands-on instruction from the veterinary staff on the appropriate techniques for handling mice and the administration of the anesthetic agent.

16. Place the anesthetized mouse on its side with the test ear facing up on the heating table or blanket. Use small, curved forceps to gently pull up on the pinna while carefully inserting the probe microphone. Slightly rotate the probe, as if turning a screw, as it is inserted into the ear canal.

    In Figure 8.21C.2, a Codman self-retaining surgical retractor is shown holding the probe. The method works well, but the tubing that connects the probe to the speakers is actually sufficient to hold the probe. A good probe fit is essential for obtaining repeatable results and the check-fit capability described above greatly facilitates probe placement. When the probe is seated properly, the fit is such that one can pull up on the probe and literally lift the head off the table.

    Depending upon the mouse or mouse strain, anesthetics may have variable results in that some mice may be unusually "light" and show muscle or vibrissae twitching, or display abnormal respiratory movements. This type of biologic noise can interfere with DPOAE measurements, but as higher frequencies are tested, this becomes much less of a problem. Robust mouse DPOAEs can typically be measured from ~6 kHz to at least 54 kHz, which is the upper limit of f_2 frequencies that the authors' system can test. Because room noise and most biological noise is <2 or 3 kHz, sound isolation is much less important for DPOAE testing in mice than in species with lower frequency hearing.

Figure 8.21C.5 *(at left)* Example of common ways DPOAEs data can be analyzed and displayed. In the left column, mean ($n = 10$) DP-grams (DPOAE level in dB SPL) are plotted as a function of the f_2 frequency for a particular tone pair evoking the emission. These plots show DPOAE levels for FVB/NJ mice obtained for three levels of primary-tone stimulation at 75 (top), 65 (middle), and 55 (bottom) dB SPL. The plots show before (solid squares) exposure, immediate (open circles) exposure, 1 day (open triangles) following exposure, and 7 days (open diamonds) following exposure to a 1-hr, 105-dB SPL octave band of noise (OBN) centered at 10 kHz (box at lower left). These plots show average DPOAE levels without any transformation of the raw data. Higher levels of stimulation at, e.g., 75 dB SPL, evoke larger DPOAEs (top left) than those produced by the 65-dB SPL (middle left) primaries. Lower levels of stimulation are frequently more sensitive to the effects of damage than higher levels of stimulation. For example, at 1 day postexposure (open triangles), successively lower primary-tone levels show less recovery, especially above 20 kHz. A number of peaks and valleys in the data are clearly evident with an especially prominent dip ~15 to 17 kHz in the 55-dB SPL plot (lower left). The prominent notch observed over this frequency range is clearly a calibration problem in the system due to standing waves that are present over this particular frequency region in the mouse ear canal. Although the authors have made several attempts to correct for this notch, it has been extremely difficult to consistently eliminate. For this reason, the authors prefer to use fixed calibrations rather than an in-the-ear procedure, where every mouse gets slightly different levels of stimulation, depending on the particular characteristics associated with that ear. The graphs in the right column show these same data plotted as differences from the preexposure baseline. The dashed line at the '0' on the ordinate indicates no change from preexposure levels. Here, it is easier to appreciate the loss patterns for the various primary-tone test levels. For example, for 75-dB SPL primaries (top right), an orderly recovery occurred from essentially NF levels immediately postexposure, to almost full recovery at 7 days postexposure, with recovery about halfway for the in-between period of 1 day after the noise trauma. Solid vertical bars indicate ±1 SD for baseline DPOAEs, while gray vertical bars show variability (±1 SD) immediately postexposure. Stippled gray line in lower portion of each plot shows the NF of measurement system.

17. Once the probe is correctly positioned, begin DPOAE testing. Depending upon the software, select the appropriate file or manually enter the instructions to begin collecting a DP-gram. Have the computer present each f_2 frequency for ~5 sec of test time so that a 10 points/octave DP-gram from 6 to 54 kHz comprising four octaves is completed in ~3.5 to 4.0 min.

This extremely short test time to obtain a frequency profile of OHC function is what makes DPOAEs testing so attractive. As more primary-tone levels are tested, the test time is substantially increased. Testing with primary-tone levels of 55, 65, and 75 dB SPL is usually adequate to give good, sensitive measures of the treatment effects being studied.

Analyze and interpret data

18. Average the results of individual mouse DP-grams to show the effects of a treatment for various treatment groups and perform standard statistical analyses between subject groups. Compare groups with a repeated measures analysis of variance (ANOVA) with f_2-test frequencies as the repeated measure.

Examples of DP-gram data showing the effects of exposure for 1 hr to a 105-dB SPL octave band of noise (stippled bar along abscissa) are shown in Figure 8.21C.5.

Because of the peaks and valleys inherent in DP-grams and the fact that different levels of stimulation produce DPOAEs that have considerably different sound pressures in the ear canal, it can be difficult to appreciate treatment effects from plots based upon the raw DPOAE data. One way to make it somewhat easier to observe the effects of a treatment is to use difference scores (step 19).

19. Convert DPOAE levels to reflect differences between pretreatment and posttreatment levels. For DPOAEs, subtract preexposure DPOAE levels from postexposure levels to yield negative values, if the treatment produced a reduction in DPOAE magnitude.

Figure 8.21C.5 shows performing this "difference" procedure on the data in the left column results in the right column.

COMMENTARY

Background Information

Otoacoustic emissions (OAEs) are defined as sounds generated by the cochlea that can be measured in the outer ear canal (Kemp, 1978, 1979a,b). Four forms of OAEs are recognized on the basis of the types of acoustic stimuli needed to elicit them. Spontaneous OAEs, or SOAEs, are present virtually continuously in the absence of deliberate acoustic stimulation. Of the remaining three OAE forms, all of which are evoked by particular categories of stimuli, transiently evoked OAEs, or TEOAEs, are elicited by brief acoustic stimuli, such as clicks and tonepips; stimulus frequency OAEs, or SFOAEs, are evoked by a continuous, low-level pure tone; and DPOAEs are elicited by two simultaneous, long-lasting pure tones.

Exactly how OAEs arise and how they are propagated in the cochlea is still a matter of debate. Recent experimental and theoretical findings (Shera and Guinan, 1999; Knight and Kemp, 2000, 2001) suggest that there may be two mechanisms of OAE generation. Thus, SOAEs, TEOAEs, and SFOAEs may arise from linear reflection from impedance discontinuities (e.g., out of place OHCs) distributed along the cochlear partition (reflection emissions), while DPOAEs likely result from nonlinear distortion (distortion emissions). The bulk of evidence now suggests that OHCs are capable of electromotile activity (Brownell et al., 1985; Brownell, 1990), with the current consensus being that OHC motility is due to the receptor-potential-initiated movements of motor molecules called prestin that are embedded in the lateral membrane of the OHC (Zheng et al., 2002). The existence of OAEs provides evidence that the cochlea is an active participant in the processing of acoustic signals in that movements of the OHCs probably act to enhance the sensitivity, frequency tuning, and temporal aspects of the vibration of the cochlear partition. In current theories of cochlear function, the OHCs are considered to act as a "cochlear amplifier" in the form of a biomechanical feedback system that sharpens the peak of the traveling wave (Davis, 1983). With OHC damage, the exquisite sensitivity,

sharp tuning, and fine timing of basilar membrane vibrations are greatly reduced and the passive mechanical analysis and poor tuning of the traveling wave noted by Bekesy (1960) predominate. OAEs are, then, either produced or influenced as a byproduct of this amplification process.

It is well established that if the OHCs are damaged, then the sharp tuning, fine sensitivity, and excellent timing of the cochlea are compromised and OAEs are reduced or absent. In the case of reflection emissions, the peak of the traveling wave (Shera and Guinan, 1999) may play an important role as a source of filtering for these emissions. Hence, OHC damage may greatly affect the reflection-type OAEs. On the other hand, DPOAEs appear to be generated in the nonlinear aspects of the OHC transduction process, probably involving the stereocilia of the OHCs. DPOAEs that are primarily generated by a subcellular process that is different from somatic electromotility explains, in part, why DPOAEs evoked by high-level primaries persist following the administration of the ototoxin furosemide (Whitehead et al., 1992b; Mills and Rubel, 1994). During such a reversible ototoxic state, it is likely that the OHC nonlinearity based on electromotility is not overly damaged. However, it is also probable that the nonlinear aspects of the transduction process involving the stereocilia are injured, so that the driving voltage across the OHC that is normally associated with the receptor potential is reduced. Without this sufficient gain, the associated basilar-membrane vibration would be missing and thus threshold-related DPOAEs in response to low-to-moderate primary-tone levels would be absent. However, for high-level primary tones, this lack of gain is overcome and suprathreshold DPOAEs can be observed. A recent study (Liberman et al., 2004) using mutant mice lacking prestin, the molecular motor responsible for OHC motility, demonstrates that OHC motility is not necessary for the production of high-level DPOAEs. Additionally, Avan and associates (Mom et al., 2001; Avan et al., 2003; Carvalho et al., 2004) reached similar conclusions regarding the nature of high-level DPOAEs by studying various cochlear traumas that preferentially affected high- versus low-level emissions.

As mentioned previously, DPOAEs are one of the easiest OAEs to measure to determine the cochlear frequency place damaged after a particular treatment. This capability is very important in most animal investigations of cochlear function. Another significant consideration is that DPOAEs are the only OAE that can be easily evoked in most laboratory animals (Whitehead et al., 1992a). DPOAEs are also very large in most rodents thus making them the OAE of choice for animal experimentation for investigators interested in obtaining a simple and rapid assessment of OHC function.

DPOAEs are defined as acoustic energy in the ear canal arising from the nonlinear interaction of two simultaneously applied pure tones within the cochlea (Kemp, 1979a). In other words, when two tones interact in a nonlinear system (not a straight line function), new frequencies are produced. This is a physical phenomenon that is not necessarily unique to the ear. For example, turning up the volume of an amplifier too high drives a speaker out of its linear operating range thus resulting in audible distortion. In the case of DPOAEs, the two evoking tones are referred to as the f_1 and f_2 primaries and the largest DPOAE occurs at the frequency equivalent to $2f_1 - f_2$. The stimulus protocol used most commonly to elicit the $2f_1 - f_2$ emissions incorporates a frequency ratio (i.e., f_2/f_1,) somewhere between 1.20 and 1.25 and usually equilevel primaries (i.e., $L_1 = L_2$).

Based on theoretical considerations and the findings of systematic research (Brown and Kemp, 1984; Martin et al., 1987), it is assumed that the generation of the $2f_1 - f_2$ DPOAE occurs primarily at the frequency position along the cochlear partition where the f_1 and f_2 forward-traveling waves maximally overlap. This situation is shown in Figure 8.21C.5 where the region of DPOAE generation is indicated by the cross-hatched area associated with the apical portion of f_2. This particular frequency region has often been represented as the geometric mean frequency of the two primaries [i.e., $(f_1 \times f_2)^{0.5}$], but recently there is a trend to use the f_2 frequency as the point of DPOAE generation based upon theoretical and practical reasoning (see Kemp, 2002). A common criterion for confirming the detection of the DPOAE is that its magnitude be at least 3 dB above the average level of the NF sampled at several frequencies surrounding the emission frequency (e.g., the mean of the NFs at the DPOAE $\pm$ 50- to 100-Hz frequencies). DPOAEs can be obtained as response/growth or input/output functions, measured at a particular frequency, in which emission magnitude is registered as a function of systematic increases in primary-tone levels (e.g., in 5-dB steps, from 25 to 75 dB SPL). However, the method of recording DPOAEs, a DP-gram in which emission levels in response to constant-level

primary tones (e.g., at $L_1=L_2=65$ dB SPL) are measured as a function of regular increments in stimulus frequency, is most popular for animal studies aimed at relating treatment effects to cochlear function in a frequency-specific manner. DP-grams are usually evoked by primaries with f_2 increments from 5 to 10 points per octave, depending on the desired frequency resolution.

DPOAEs, as well as the other OAEs, have a number of characteristics that make them ideal tests of the auditory periphery's ability to process sound. That is, they (1) are objective and easily measured using noninvasive methods and simple subject preparation; (2) require brief examination periods; (3) have high test/retest reliability; (4) are preneural in origin in that they are specifically generated by the OHC system, which is well known for its sensitivity to the adverse effects of cochlear traumas; and (5) are present in essentially all normal ears, but are reduced or absent in ears with OHC damage, thus allowing the distinction between normal and pathological function to be determined (Probst et al., 1991; Lonsbury-Martin et al., 1993).

Although there is ample commercial instrumentation available for measuring human DPOAEs, there is little, if any, commercial equipment specifically designed and marketed for animal studies, especially for subjects like mice that have high-frequency hearing capabilities. Some commercially available equipment have a maximum upper frequency limit of ~32 kHz that could be used for testing mice DPOAEs up to 32 kHz, assuming that the emissions probe can be adapted to fit their tiny ear canals. This frequency represents somewhere between 60% and 75% of the mouse cochlea (Ou et al., 2000), depending upon the particular frequency-position transform for the mouse. In many situations, this frequency range may be adequate. However, for disorders that preferentially affect the basal, high-frequency end of the cochlea, such as age-related hearing loss or ototoxicity, it may be desirable to be able to test considerably beyond 32 kHz to detect the earliest possible change in OHC function. This requires the researcher to assemble specialized stand-alone equipment, because until recently, most computer soundcards were not able to generate stimuli much higher than 20 kHz. Commercially available equipment that can exceed this limit is based upon special-purpose computer boards. However, even with high-frequency stimulus generation capabilities, the software available for this equipment is not necessarily optimized for DPOAE measurement. As a result, it is evident from the existing mouse DPOAE literature that practically every laboratory uses different equipment for testing mouse DPOAEs.

In spite of these technical limitations, there are a surprising number of publications in which mouse DPOAEs have been employed as a measure of cochlear or OHC function. One of the earliest studies of mouse DPOAEs examined mice with selective inner hair cell loss or absence of the organ of Corti to determine if OHCs were necessary for the generation of DPOAEs (Horner et al., 1985). Nearly 10 years later, Huang and co-workers (Huang et al., 1995, 1996, 1998) used DPOAEs as a means to phenotype inbred strains of mice with hearing defects. This particular application for mouse DPOAEs is still an important one today. DPOAEs have also proven very useful in studying age-related hearing loss that results from a genetic mutation common to a number of inbred mouse strains (e.g., Parham, 1997; Jimenez et al., 1999; Parham et al., 1999, 2001). Because of the sensitivity of OHCs to a variety of cochlear insults, mouse DPOAEs have been used to study the effects of sound exposure or of ear susceptibility/resistance to noise stimuli (Yoshida and Liberman, 2000; Jimenez et al., 2001; Vazquez et al., 2001, 2004; Candreia et al., 2004). Recently, measurement of DPOAEs in mutant mice has been used to help understand the role of the tectorial membrane in cochlear mechanics (Lukashkin et al., 2004). Thus, measurement of DPOAEs in mice can be useful for unraveling basic questions concerning how the ear operates or as a sensitive monitor of OHC function. However, it is important to stress that setting up a measurement system for DPOAEs in mice is somewhat difficult to implement due to the lack of commercially available equipment from a single source.

Critical Parameters

Success in using DPOAEs as a measure of cochlear function requires an understanding of some of the critical parameters needed to evoke robust emissions. An excellent discussion of many of these variables with respect to the mouse can be found in Parham et al. (2001). Some of the more critical aspects involved in the production of DPOAEs are reviewed here.

Influence of f_2/f_1 ratio

Generation of distortion products occurs when two tones are introduced simultaneously into a nonlinear system. In the case of DPOAEs, the nonlinearity arises in the OHC transduction process. When tones are presented to the ear, they are represented as traveling-wave envelopes on the basilar membrane (Fig. 8.21C.6). In normal ears, these envelopes have a sharp peak with a very steep apical cutoff (low-frequency side) and a much more gradual tail extending in the basal direction (high-frequency side). For two tones to interact, their traveling-wave envelopes must overlap so that they are both stimulating the same nonlinearity, otherwise no distortion would be produced. In other words, the low-frequency (f_1) and high-frequency (f_2) tones must be close enough on the basilar membrane to optimize their interaction. This occurs in many species at an optimal f_2/f_1 ratio around 1.2 to 1.25 (Harris et al., 1989). When the primaries are moved apart, the DPOAE level decreases. Unexpectedly, when the two tones are too close together, DPOAEs also decrease. Several explanations for this reduction in DPOAE level have been proposed, but none have been experimentally proven. Maximal distortion is also produced when the inputs to the nonlinearity are equal in level. Because of the saturating nonlinear aspects of basilar membrane excitation at the characteristic frequency (in this case f_2), the level of f_2 (L_2) must be lower than the level of f_1 (L_1) to optimize DPOAEs (Whitehead et al., 1995a,c; Kummer et al., 2000). This difference is on the order of 5 to 10 dB and becomes negligible for higher-level (75-dB SPL) primaries. In animals with large DPOAEs, equilevel primaries are adequate for most studies. Besides the overall lowering of the primaries in general, lowering L_2 with respect to L_1 can further increase sensitivity to damage (Sutton et al., 1994; Whitehead et al., 1995a).

Place of DPOAE generation

One of the more confusing aspects of understanding DPOAEs is relating the DPOAE frequency to the site of cochlear damage. For example, it is not intuitively obvious what frequency place in the cochlea is damaged when the $2f_1$-f_2 DPOAE near 15 kHz is reduced from normal. In this case, as noted in Figure 8.21C.5, the three frequencies of concern are the f_1 at 20.2 kHz, the f_2 at 25.3 kHz, along with the 15-kHz DPOAE frequency. Fortunately, a number of studies have addressed the issue of where DPOAEs are generated, and they indicate that the $2f_1$-f_2 DPOAE is produced either between the frequency places represented by the f_1 and f_2 primary tones, or near the place of the f_2 tone (Brown and Kemp, 1984; Martin et al., 1987, 1998).

Because of basilar membrane considerations described above (i.e., very sharp apical cutoff), the only place where the two tones can interact is at f_2 place or basal to this frequency. Consequently, it is generally agreed that the DPOAE is generated very near the f_2 place and that decreases in a particular DPOAE should be plotted at the corresponding f_2 frequency (Kemp, 2002).

Effects of primary-tone levels

A final critical parameter is the choice of absolute levels of the primaries that are to be employed. Early research showed that when the loop diuretics ethacrynic acid or furosemide (known poisons to the stria vascularis that generates a battery across the OHCs) were administered, DPOAEs evoked by low-level tones were abolished while DPOAEs produced by higher-level tones were essentially unaffected. These findings led to the notion that there were two sources, a high-level and a low-level source, for the generation of DPOAEs (Whitehead et al., 1992b; Mills and Rubel, 1994). More recent studies have led to a new interpretation of these puzzling results. Namely, DPOAEs are viewed as being generated by a single nonlinearity involved in OHC transduction (Lukashkin et al., 2002). The loss of low-level DPOAEs in the ethacrynic acid/furosemide studies took away the voltage necessary for OHC motility and amplification of basilar-membrane motion. However, when high-level tones were presented, this deficit was overcome and normal DPOAEs were produced. In the example of noise exposure in Figure 8.21C.5, note that DPOAEs produced by high-level primaries at 75 dB SPL were drastically affected. In fact, immediately after the noise exposure DPOAEs evoked by 75-dB SPL primaries were essentially reduced to the NF, just like those produced by much less intense tones. This can be explained by the fact that, unlike the loop diuretics, noise exposure has very detrimental effects on the OHC transduction process. That is, noise may damage OHC tip links or transduction channels, or affect the stiffness of the OHC stereocilia or all of these subcellular components. Thus, DPOAEs produced by higher primary-tone levels will be affected depending upon the nature of the damage to the OHC transduction process. Overall, it is important to appreciate

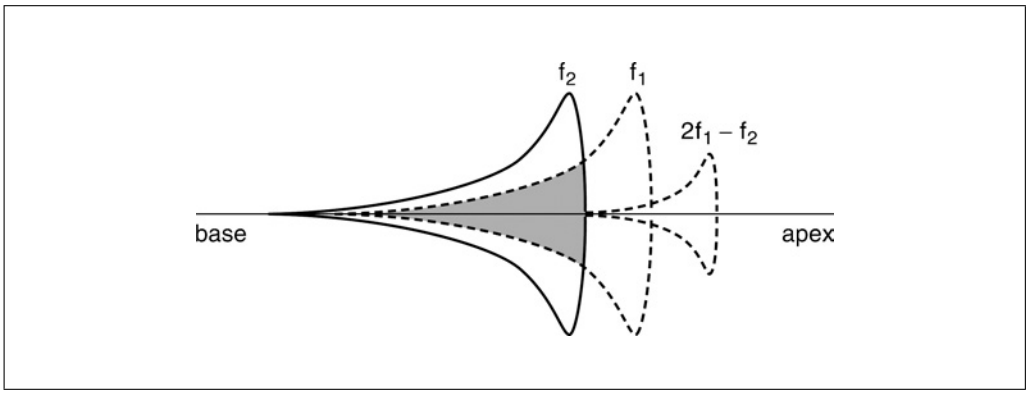

Figure 8.21C.6 Diagram depicting the traveling-wave envelopes on the basilar membrane for f_1, f_2, and the $2f_1$-f_2 DPOAE. Note that the $2f_1$-f_2 DPOAE is lower in frequency (and level) than the primary tones, but is generated where the two stimuli overlap (shaded area) basal to the DPOAE characteristic frequency place. Because of the sharp apical cutoff of the f_2 traveling wave, the DPOAE can only be generated at the f_2 frequency place, or basal to this region.

that depending upon primary-tone level, all aspects important to OHC transduction, including cochlear amplification, will manifest into alterations in DPOAEs. It is also important to realize that even a complete loss of DPOAEs does not necessarily mean that the OHCs are missing. However, if the OHCs are destroyed, then measurable DPOAEs will be absent.

Troubleshooting

Excessive noise when trying to measure DPOAEs

Excessive noise often results from a poor electrical connection somewhere in the system. However, it may arise from animal movements such as irregular breathing or twitching. To troubleshoot this problem, place the probe in the syringe cavity and determine if the noise persists. If the noise still exists, then the problem is in the measurement system. This most often occurs on the output side of the microphone. First, be sure the microphone batteries are fully charged because low batteries can lead to increased noise. If this fails to correct the problem, remove connections from the microphone to other equipment and spray with electrical contact cleaner. Reassemble the connections and test the system again using the syringe cavity. If these measures fail to correct the problem, the microphone is not working properly. Overall, these microphones are surprisingly robust and should be returned to the manufacturer as a last recourse. However, it is important to be aware of the fact that emissions microphones are extremely sensitive to, and can be damaged by, any moisture that enters the microphone port. Consequently, emissions microphones should be stored in a desiccator to prevent damage from moisture in the environment when not in use, especially in humid climates. If possible, it is very desirable to have an extra microphone available to test the likelihood that the fault lies in the microphone or associated preamplifier. Additionally, if the microphone becomes damaged for any reason, replacement times can be considerable thus preventing research from progressing or ruining an experiment in progress.

Correct primary-tone levels cannot be achieved

Inspect the sound-delivery tubes to be sure the connections are secure and tight. Also, the sound exit ports and microphone input port must be checked to be certain they are not blocked with cerumen or other debris. Next, the steps described above should be used to check the output side of the equipment. This includes making sure the attenuators are set correctly. If the loss in primary tones is clearly asymmetrical, i.e., one tone is lower than the other, the low side of the output is probably the problem. Be certain that applying too much voltage to the input of a speaker has not accidentally damaged the transducer. If both signals are lower than desired and checking the output fails to correct the problem, then the microphone side of the system may be malfunctioning and should be checked as described above.

Excessive distortion obscures the DPOAE being measured

Excessive distortion is most often caused by overloading the system at some point, usually the input to the microphone or the soundcard. Because designers of commercial spectrum analyzers anticipated this problem, these instruments will change the input gain (autorange) to prevent overloading. Overloading and associated distortion can occur if an

Table 8.21C.1 Sample Microphone Correction and Speaker Calibration File

Microphone correction frequency (kHz)	Correction (dB)	Speaker calibration frequency (Hz)	Max out f_1 (dB SPL)	Max out f_2 (dB SPL)
0.1	0.0	0.1	99.3	100.5
0.2	0.0	0.2	109.0	109.5
0.3	0.0	0.3	113.0	112.5
0.4	0.0	0.4	115.0	115.0
0.5	0.0	0.5	115.7	116.0
0.6	0.0	0.6	115.3	116.0
0.7	0.0	0.7	114.7	115.0
0.8	0.0	0.8	114.0	114.5
0.9	0.0	0.9	113.3	113.5
1.0	0.0	1.0	113.3	113.5
1.1	0.0	1.1	113.3	113.0
1.2	0.0	1.2	113.7	113.0
1.3	0.0	1.3	114.0	113.0
1.4	0.0	1.4	114.0	113.0
1.5	0.0	1.5	114.0	113.0
1.6	0.0	1.6	113.0	113.0
1.7	0.0	1.7	112.3	112.5
1.8	0.0	1.8	111.7	112.0
1.9	0.0	1.9	111.0	112.0
2.0	0.0	2.0	111.0	111.0
2.1	0.0	2.1	111.0	111.0
12.4	5.1	12.4	134.7	127.5
12.5	5.2	12.5	133.7	126.5
12.6	5.3	12.6	132.7	125.5
12.7	5.5	12.7	131.3	124.5
12.8	5.6	12.8	130.3	122.5
12.9	5.7	12.9	129.7	121.0
13.0	5.8	13.0	129.0	120.0
13.1	6.0	13.1	128.7	119.0
13.2	6.1	13.2	128.0	118.5
13.3	6.2	13.3	127.3	118.5
13.4	6.4	13.4	126.3	118.5
13.5	6.5	13.5	126.0	119.0
13.6	6.6	13.6	125.3	118.5
13.7	6.7	13.7	124.3	117.0
13.8	6.9	13.8	124.0	115.0
13.9	7.0	13.9	123.3	114.0
14.0	7.1	14.0	123.0	113.0
14.1	7.2	14.1	123.0	112.0
14.2	7.3	14.2	123.0	112.0
14.3	7.5	14.3	122.3	112.0
14.4	7.6	14.4	122.3	112.5
14.5	7.7	14.5	122.3	120.0

attenuator malfunctions or if the calibration is incorrect due to the standing-wave problems described above. Check attenuators for problems and, if they are operating correctly, be sure calibration tables are not requesting too little attenuation. A good clue to this latter problem is if nearby attenuation values in the calibration table are much higher than the one in question, suggesting that this frequency is the source of overloading.

Anticipated Results

Once the appropriate equipment is in place, measuring DPOAEs is simple and straightforward. The procedure is noninvasive and no surgical procedure or training of the mouse is required. Depending upon primary-tone levels, DPOAEs are very large in the neighborhood of 35 dB SPL and, consequently, interference from ambient noise is not a major problem. If the equipment NF is around −10 dB SPL, these emissions are easy to distinguish from the NF over a considerable range of primary-tone levels. If a treatment damages the OHCs or practically any aspect of OHC transduction, then it should be apparent in a change in DPOAEs from baseline measures. It should be noted that DPOAEs (Fig. 8.21C.5) can, in some cases, increase following cochlear damage. This paradoxical increase presumably reflects a change in the nonlinearity that is the source of DPOAE generation, either in the form of a change in the operating point (the location on the transducer response curve in the absence of stimulation) or in the form (shape of the transducer response curve) of the nonlinear function (for examples, see Frank and Kossl, 1996; Fahey et al., 2000; and Sirjani et al., 2004).

Typical microphone correction and speaker calibration data are shown in Table 8.21C.1. In this example, correction factors and calibrations were determined in 0.1-kHz steps. It can be seen that microphone correction factors (second column from left) are 0.0 dB from 0.1 kHz to 2.1 kHz because in this low-frequency range the microphone response is flat. No corrections are required because the microphone is designed to have a smooth frequency response to accurately measure the sound pressure at these frequencies. In the next section of the file (12.4 to 14.5 kHz) the microphone begins to lose sensitivity and ∼5 to 7 dB must be added to obtain the correct reading in dB SPL. The middle column shows the test frequencies used for calibration of the speakers and the two columns on the right display the measured output for the f_1 and f_2 speakers. It can be seen that there are various peaks and valleys in the calibration values. For the higher frequency portion, a peak is reached ∼13 kHz for the f_1 speaker, whereas for the f_2 speaker, the peak output occurs near 12.5 kHz. Based upon these values, it is possible to apply the appropriate attenuation to achieve a constant output from each speaker as a function of frequency. Note that for the mouse, a complete file would extend from ∼0.1 kHz to 35 to 40 kHz for the microphone corrections and from 0.1 kHz to 60 kHz for the speaker outputs.

Time Considerations

It usually takes no more that 3 to 4 min to obtain a detailed DP-gram describing OHC function across ∼80% of the hearing range of the mouse. This is a quick procedure that allows one to test both ears as a type of internal control. Data from both ears can then be averaged or one ear randomly selected for statistical analysis. Most students or technicians can easily learn to reliably place the probe in the ear canal, making this procedure available to a large number of users.

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Key References

Jimenez, et al., 1999. See above.

Jimenez et al., 2001. See above.

Original peer-reviewed published articles reporting many of the details itemized in the Basic Protocol.

Kemp, 2002. See above.

Current view of the generation and propagation of otoacoustic emissions.

Parham, 1997. See above.

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Parham et al., 2001. See above.

Earlier review of research on DPOAEs in mice.

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