Behavioral Assessment of Hearing in Mice—Conditioned Suppression

The conditioned suppression procedure described in this unit involves training an animal to stop drinking from a waterspout when it detects a sound, or a difference between sounds, in order to avoid an electric shock. It is a simple procedure that takes advantage of the natural freezing response of an animal when it senses a change in its environment—the same response seen in a wary animal drinking at a waterhole. It requires little cognitive or motor skill thus making it suitable for testing animals with severe cognitive and motor impairments. This procedure has been used to assess a wide range of auditory abilities, including absolute sensitivity, intensity and frequency difference limens (thresholds), sound-localization acuity, and the ability to discriminate between two classes of sounds.

This unit provides a detailed procedure to determine the absolute sensitivity of a mouse to pure tones, i.e., its audiogram. Comments are included to describe how the procedure can be modified to test auditory discrimination.

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

Materials

Mice
Fruit juice (e.g., cantaloupe/pear juice) or water
Dry mouse food

Sound-transparent test cage with waterspout
Sound-proof room
Loudspeakers for transducing pure tones from 1 to 100 kHz (e.g., a tweeter, available from Panasonic, for frequencies of 4 kHz and higher; a 15-in. woofer, available from Pioneer, mounted in a rattle-free enclosure for lower frequencies)
Television camera and monitor
Electronic lick circuit or touch switch (for electronic diagrams of lick circuits, see Weijnen and Mendelson, 1977)
Syringe pump (capable of dispensing at 5 to 20 ml/hr and clear plastic tubing, e.g., $^1/_4$-in. or 6.4-mm i.d., to connect the syringe to the waterspout; e.g., model NE-1000, New Era Pump Systems)
Sound-attenuating box, optional
Computer for control of stimuli delivery, measurement of behavioral reponse and calculation of performance score
Shock generator and mechanical relays for switching the shock (e.g., shock generator can be a high-voltage transformer, such as those found in inexpensive AC fence chargers, e.g., the Red Snap'r model 33B, plugged into a continuously variable transformer to control the voltage, e.g., model 171, Staco), shock level $\leq$1.25 mA
Tone generator covering the range from 1 to 100 kHz (e.g., model SR 770, Stanford Research Systems)
Rise-fall gate (e.g., model S84-04, Coulbourn)
Attenuator (e.g., model S85-08, Coulbourn)
Variable audio bandpass filter (e.g., model 3202, Krohn-Hite), optional
Audio amplifier (e.g., model D-75, Crown)
Oscilloscope for monitoring the electrical signal (e.g., model TDS 210, Tektronix)

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Sound-measuring microphone, preamplifier and/or amplifier covering the range from 1 to 100 kHz (e.g., conditioning amplifier model 2690, preamplifier model 2669, microphone adapter model UA0035, and $\frac{1}{4}$-in. microphone model 4939, all available from Brüel & Kjaer)

Microphone calibrator (e.g., sound level calibrator model 4231, Brüel & Kjaer)

Spectrum analyzer covering the range from 1 to 100 kHz (e.g., model SR 770, Stanford Research Systems)

Set up initial equipment

1. Place the sound-transparent test cage with waterspout in the approximate center of the sound-proof room (i.e., away from the walls). Place a loudspeaker capable of producing tones from 8 to 32 kHz (the range of best hearing for mice) ~1 m in front of the cage at an elevation of 0° to 15° relative to the head of the animal when it is drinking from the waterspout.

   The cage shown in Figure 8.21D.1 is 14 × 8 × 10 (h)–cm, constructed of 0.5-in. (1.27 cm) wire mesh (hardware cloth) with the top hinged for placing and removing the animal. The cage is constructed in-house with the top and bottom attached to the sides with nylon cable ties. The waterspout is a 2-mm diameter brass tube, topped with a curved 5 × 8–mm lick plate attached at ~45° angle. It is mounted vertically so that it projects up through the cage bottom with the top ~2 cm above the cage floor, low enough not to affect the sound reaching the animal's ears. A wire mesh fence, approximately the height of a mouse's shoulder, is permanently secured inside the cage with nylon cable ties to ensure that an animal will face directly forward when licking the spout. A 2-mm-thick sponge, sandwiched between the floor of the cage and a piece of wire mesh, is moistened to provide good electrical contact with the animal's feet, as the electric shock is transmitted between the waterspout and the cage floor. Animals with tremors tend to make intermittent contact with the waterspout causing the amount of shock they receive to vary from trial to trial thus making their performance erratic (Koay et al., 2002). To ensure a constant shock level in these animals, it necessary to deliver the shock to their feet through a

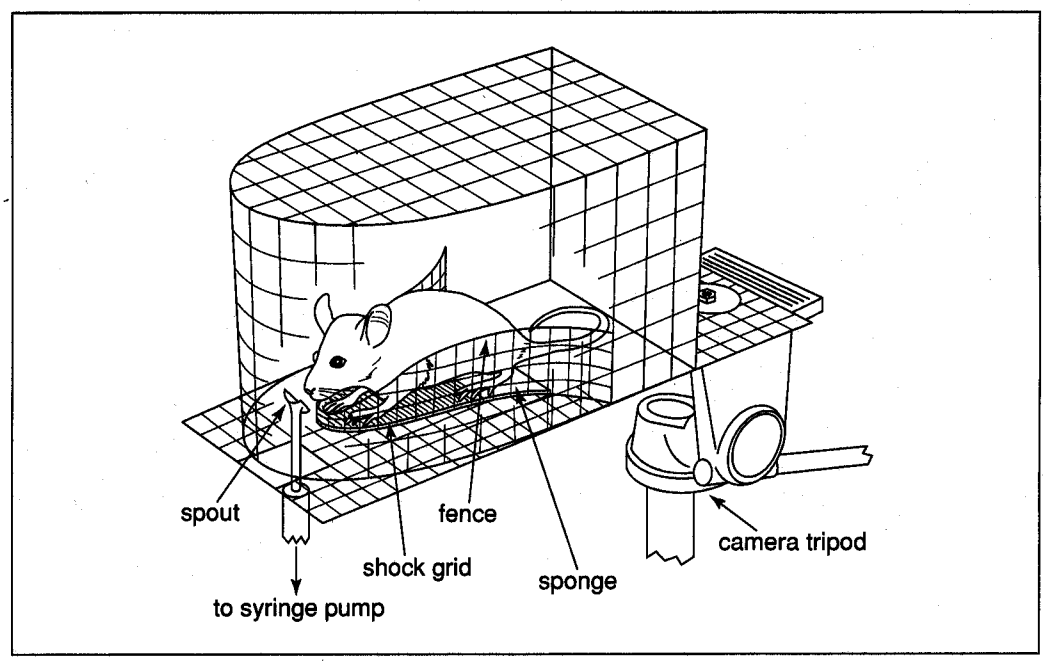

Figure 8.21D.1 Cage for testing hearing in mice. Requiring the animal to drink continuously from the waterspout positions its head within the sound field. The curved sides of the lick plate and the fence inside the cage help line the animal up facing the loudspeaker. Note that the lick plate on the waterspout is small and is positioned below the animal's ears so that it does not block the sound. Mild avoidable shock is delivered between the waterspout and cage floor and, in some cases, through a grid floor. (Modified from Koay et al., 2002.)

grid floor in which the shock is delivered through alternate floor bars. Such a grid can be constructed of 22-G copper or stainless steel wire inserted onto a perforated circuit board with ~0.1-in. (~2.5-mm) spacing, with the wire running the width of the floor on which the animal stands (see Fig. 8.21D.1). The cage is placed on a tripod ~1 m above the floor. A 25-W light is mounted ~0.5 m below the cage and is turned on whenever the electric shock is on. The bulb itself need not be within view of the mouse, but its light should be easily visible in the dimly lit chamber.

Testing is conducted in a room sufficiently insulated to keep out sounds in the range of mouse hearing which, at a level of 60 decibels (dB) sound pressure level (SPL re 20 μNewtons/m^2), is from 2 to 88 kHz. The inner walls should be lined with acoustic or egg-crate foam and the floor at least carpeted to reduce sound reflection. Equipment in the room should be checked to ensure that it does not emit sounds audible to mice (e.g., syringe pump, lights, television cameras). If audible sounds are detected, they must be eliminated by sound-absorbing coverings or by substituting equipment that does not emit sounds audible to mice. Any extraneous sounds in the room could prevent the mice from hearing the test signals.

2. Place the television camera so that (1) it provides a good view of the mouse's mouth when it is drinking from the waterspout, (2) the animal can be seen wherever it is in the cage and, (3) the camera is not in the sound field (i.e., place it behind the loudspeaker, off to one side, or above the test cage).

3. If using a lick circuit (as opposed to a touch switch), connect one lead to the waterspout and the other to the cage floor. Connect the syringe pump to a relay controlled by the lick circuit so the pump operates whenever the mouse makes contact with the spout.

   Recommended syringe size is 10-ml, although larger and smaller sizes can be used. If the pump is inside the sound-proof room, it should be placed in a box lined with insulation to attenuate the sound of its operation.

4a. If using fruit juice, put the syringe pump in a sound-attenuating box and place it beneath the test cage to keep the tubing to the waterspout short, as it must be rinsed daily with water.

   A cantaloupe/pear juice mixture works well; mice do not like apple juice.

4b. If using water, place the syringe pump outside the sound-proof room and run the tubing through the wall. Use distilled water, if available, as it will reduce the growth of algae in the water line.

Train mouse to maintain steady contact with the waterspout

5. Weigh the mouse to determine its ad lib weight and remove its water ~24 hr before it is to be trained, but continue to give it free access to dry food.

   The weight of the animal will be recorded just before each experimental session and compared with its ad lib weight to monitor its health and deprivational state. Healthy adult mice may lose 20% body weight over the first five to eight sessions, and regain some of that weight after they have learned the task; young mice will continue to grow and may eventually exceed their original ad lib body weight.

6. Set the syringe pump to 20 ml/hr; run it until there are no air bubbles in the line and drops can be seen coming out and collecting on the spout (the spout should be able to hold a drop of water but larger amounts should drip off). Place the mouse in the test cage and allow it to explore and drink for 15 to 20 min (many animals will not drink much the first day and a 35- to 40-g mouse can maintain its body weight on as little as 1 to 2 ml/day). Do not provide water outside the test cage in order to maintain motivation. Gradually reduce the flow rate over the next few daily sessions until the animal is drinking steadily for 20 min or more, typically at a flow rate of 7 ml/hr.

An experienced researcher using a test cage that has been adjusted to yield optimal performance may be able to get a naïve animal to drink steadily by the third session, but five to ten sessions may be more realistic for inexperienced researchers using untried equipment.

It is important to get the animal to drink steadily and minor adjustments may have to be made. For example, an animal may not drink steadily if it cannot comfortably drink from the waterspout, if the flow rate is too low, or, in the case of fruit juice, if the concentration is too strong or the juice otherwise not to its liking. In addition, a mouse given supplemental water or wet food in its home cage will not be sufficiently thirsty to drink much in the test cage.

It is best if an animal maintains continuous contact with the waterspout while drinking, which some animals do by putting their mouth on the lick plate and others do by grasping the waterspout with a front paw. However, some mice may contact the spout only with their tongue, and those with motor disorders may have tremors. In such cases, the lick circuit opens and closes with each lick causing the syringe pump to pulse and the flow rate to vary. Sometimes this can be fixed by adjusting the position of the waterspout and the angle of the lick plate. At other times, it is necessary to connect the lick circuit to a timer or timing circuit that puts out a continuous pulse to the syringe pump as long as the breaks in contact do not exceed 100 msec.

Mice are capable of drinking steadily and listening for sounds for up to 30 min in a single daily session. This is adequate time to permit enough trials on which to base a threshold. Sessions should be at least 15 min long as that is the minimum usually necessary to obtain a reliable threshold.

7. Adjust the position of the waterspout and fence so the animal faces forward and maintains its head in a fixed position in the sound field.

   In the early stages of training, some mice may climb or stand in undesirable positions. This can be eliminated by stopping the flow of water or juice to the spout until the mouse stands in the required position—this is most easily accomplished by the addition of a manual cutout switch controlled by the experimenter. The cutout switch is usually a hand-held pushbutton switch that momentarily interrupts the operation of the pump when pressed.

Set up behavioral test equipment

8. Connect the output of the lick circuit (or if a timer is used to ignore short breaks in contact, connect the output of the timer) to the computer input line.

   The computer will turn on the auditory stimuli, record a response of the animal, and calculate its performance score. It should have one input line for the response and two output lines, one to turn on the tone and the other to turn on the electric shock. (Additional output lines may be needed when testing the ability to discriminate between different sounds.) In writing the computer program to control the experiment, it is important to confer with someone experienced in the method of conditioned suppression for advice.

9. Configure the computer output to turn on the tone, which can either be on steady or pulsed by external timing circuitry (e.g., 400 msec on, 100 msec off).

10. Configure the computer output to turn on the shock and shock signal light. Run the shock line from the computer to a relay that disconnects the lick circuit and connects the shocker to the waterspout and cage floor—the lick circuit must be disconnected when the shock is applied or it may be damaged. If a high-voltage transformer is used as the shocker, connect a second relay to provide 110 VAC to the input of the variable transformer into which the high-voltage transformer is plugged so that there is no high voltage present except when the shock is being delivered. Finally, include a third relay to turn the shock signal light on and off with the shock.

    The shock will be delivered between the waterspout and cage floor (and, in the case of a grid floor, between alternate floor bars or wires).

Program computer for behavioral testing

11. Program the computer to present a series of 2-sec trials in which a tone is presented during ~22% of the trials ("warning" trials), with the remaining trials consisting of silence ("safe" trials). For warning trials, present each tone for 2 sec followed by a brief (0.3-sec) avoidable and escapable shock. Allow a 1-sec intertrial interval between trials during which time the computer updates the mouse's score and determines if the animal is still in contact with the waterspout. Allow an additional 3-sec interval following warning trials to give an animal time to return to the waterspout.

    The computer determines if the mouse is in contact with the spout before each trial and does not initiate the trial unless the animal is in contact.

    In absolute threshold testing, the mouse is unaware of the progression of safe trials as these are silent trials. Warning trials are signaled by the onset of a tone, which causes a well-trained animal to break contact with the waterspout, thus avoiding the shock that is delivered through the waterspout. (If the shock is delivered through a grid floor, the computer is programmed not to turn on the shock if the animal breaks contact with the spout during a warning trial.) The light from the shock light indicates when the shock is on and turning it off indicates to the animal that it is safe to return to the waterspout.

12. Present warning trials randomly from one to five trials after the last warning trial. Construct a quasi-random trial sequence so that there is an equal probability of a warning signal being presented on each trial (e.g., Table 8.21D.1).

    It is not appropriate to determine warning trial presentation by randomly choosing a number from 1 to 5 because this will cause the probability of a warning trial to vary from 1/5 for the first trial after a warning trial to 1/1 for the fifth trial. This, in turn, will cause animals to be less likely to respond to a tone that occurs in the first few trials following a warning trial and more likely to respond when the tone occurs after four or five safe trials.

13. Score the trials by having the computer look ten times during the last ~200 msec of a trial to determine if the animal is in contact with the spout. Each trial is scored from 0 (no spout contact) to 10 (continuous spout contact) with intermediate scores indicating intermittent contact.

    Scores from 0 to 5 are typically counted as an avoidance response whereas those from 6 to 10 are not. Most scores will be either 0 or 10, and although the occasional intermediate score is usually not a concern, excessive partial scores (e.g., >10%) indicate a problem that should be fixed.

Table 8.21D.1 Example of a Distribution of Warning Trials With a Relatively Equal Probability of a Warning Trial Occurring in Each Trial Position

Trial position	Number of warning trials given in this position	Number of safe trials occurring in this position	Probability of a warning trial in this position
First	6	22	.214
Second	5	17	.227
Third	4	13	.235
Fourth	3	10	.231
Fifth	2	8[a]	.200

[a]Eight sequences of five safe trials (with no warning trial) are given so that the probability of a warning trial in position 5 is .200. All other trial sequences end with the delivery of a warning trial. Overall probability of a warning trial is ,222.

14. In signal detection terms, an avoidance response to a warning trial is referred to as a "hit" whereas a response to a safe trial is a "false alarm." Express each animal's performance as the hit rate corrected for the false alarm rate, using the following equation:

    performance = hit rate – (hit rate × false alarm rate).

    Threshold is defined as the intensity that corresponds to a performance score equal to 0.50 (usually derived by interpolation). Calculate chance performance using either the Mann-Whitney U test (e.g., Siegel, 1956) or the binomial distribution (e.g., Heffner and Heffner, 1995); a number of free and commercially available statistical packages can be found on the Internet for these calculations.

Set up acoustic equipment

The signal generation and gating setup depends upon the available equipment. The following is a description using stand-alone equipment. It is possible to obtain some of the sound-generation equipment in a combined unit, such as the Tucker Davis Technologies Psychoacoustic Work Station. However, it is important to ensure that such a unit can produce signals up to 100 kHz.

15. Connect the output of the tone generator to a rise-fall gate, which will gate the signal on and off and eliminate onset and offset transients. Because turning the tone on and off abruptly will cause onset and offset artifacts (clicks), use a 10-msec rise-fall time for frequencies of ≥1 kHz. (Generally, allow at least ten cycles of a signal to elapse during the rise and fall of the signal.)

    The onset and offset of the tone can also be synchronized to the zero crossing of the sine wave.

16. Connect the output of the rise-fall gate to an attenuator to reduce the intensity of a tone to obtain a threshold.

17. (Optional) Connect the output of the attenuator to a variable audio bandpass filter to remove any distortion or noise in the electrical signal (usually set to 1/3-octave, or less, above and below the test frequency).

18. Incorporate an audio amplifier to increase the amplitude of the electrical signal, especially if the signal must be >60 dB SPL.

    Ensure that the amplifier does not introduce noise that an animal can use to determine when a warning trial is in progress—this can be a problem with low-quality amplifiers or even high-quality amplifiers of 100 W or more. If high intensities are not needed, an impedance-matching transformer, which matches the impedance of the electrical signal (typically 600 Ω) to the loudspeaker (typically 8 to 16 Ω), may be used.

19. Connect the output of the amplifier or impedance-matching transformer to a loudspeaker.

20. Connect an oscilloscope to the line going to the loudspeaker to visually monitor the electrical signal to ensure that the signal is undistorted, i.e., that it is a pure sine wave. Visually inspect the signal first with the bandpass filter set to a wideband setting to ensure that a distorted signal is not being generated.

21. Check the linearity of the attenuator by noting the change in the voltage of the signal on the oscilloscope or a voltmeter (a 6-dB attenuation should reduce the voltage of the signal by half and a 20-dB attenuation should reduce it by a factor of 10).

    Attenuator linearity should be checked for the entire range over which it will be used. It is not uncommon for an impedance mismatch to cause an attenuator to be non-linear for

the first 15 dB of its range. This is not a problem as long as the actual attenuation value is known; one solution is to begin with an attenuator setting of 20 dB. Another potential problem is that the attenuator may not reduce the intensity of the signal beyond a certain level.

Measure acoustic signal

22. Calibrate the sound-measuring equipment with the microphone calibrator according to the manufacturer's instructions. Place the microphone in the position normally occupied by the animal's head when it is drinking and point it directly at the loudspeaker (0° incidence). Move it around to make sure that the sound field is homogeneous (±1 dB) in the area occupied by the head when the animal is drinking—if it is not homogeneous, then re-orient the loudspeaker and/or move it further away from the cage.

    The exact distance between the speaker and cage is not important as long as the sound is at an acceptable level and does not vary within the listening area for the mouse. However, it should not be so close that an animal can feel the movement of air produced by the speaker diaphragm.

23. Use the spectrum analyzer to determine if the tone contains harmonics, i.e., frequencies at integer multiples of the fundamental frequency.

    Harmonics are particularly likely when testing low frequencies at high intensities because the harmonics, which are almost always higher in frequency than the test tone, will fall into a more sensitive portion of the mouse's hearing range. Harmonics usually disappear quickly as the intensity of the signal is reduced and can be tolerated at high intensities as long as they have disappeared well before the threshold of the animal is reached—otherwise the animal's sensitivity to the harmonics rather than to the fundamental frequency may be what is measured.

24. Use the acoustic signal to check the linearity of the attenuator (see step 21).

    The small-diameter microphones used to measure the high frequencies that mice hear are generally not as sensitive as the animal, so it may not be possible to measure the faint acoustic signal at the level of a mouse's threshold. If this is the case, use the electrical signal going to the loudspeaker to verify the attenuation.

25. Use the manufacturer's free-field calibration curve for the microphone, noting whether the calibration was done with or without the protective grid, and apply any necessary corrections.

26. Finally, calibrate each day a threshold is taken. If this is not possible (e.g., if the measuring equipment is not continuously available), carefully record the initial voltage to the loudspeaker, the equipment used to generate the tone, the position of the speaker relative to the test cage, and calibrate as soon as possible.

    Although the intensity of a sound will remain constant as long as no crucial settings are changed and the loudspeaker is not overdriven, taking all precautions is recommended.

Train mouse to stop drinking when it hears a sound

The following steps describe the initial suppression training procedure.

27. Present trials with the tone and shock turned off to get an idea of how steadily the animal is working and how many warning trials can be obtained in a single testing session before the mouse becomes satiated and no longer drinks for that day.

    This step can be performed before the mouse has learned to drink steadily.

28. Choose an easily audible tone for initial conditioning, e.g., 8 kHz at ~60 dB SPL is a good choice because it is audible to normal mice as well as to humans (unless they have high-frequency hearing loss).

If working with mice suspected of having a hearing loss, a higher intensity may be necessary.

29. To determine the starting shock level, set the shock level at zero and disconnect the shock signal light. Manually turn on the shock (without a warning sound) while the animal is drinking, and turn it up until the mouse reacts to it (e.g., animals typically break contact and look at the spout when they first detect the shock).

    This is the starting level for the shock, which is just noticeable to the mouse and well below the level that it will find aversive. This shock level can be used as the starting level for subsequent animals as well.

30. Reconnect the shock light, set the shock duration to 2 sec, and let the computer present trials, including tone (warning) trials.

    Initially, the animal will break contact on warning trials because it is startled by the tone, but once it has habituated to the tone, it will drink through the warning trial, and the starting level of the shock (determined in the previous step) will probably be too low to make the mouse break contact. When this happens, turn up the shock level after each warning trial until you reach a level that causes the animal to break contact and stay off the spout as long as the shock is on.

31. Once the animal has learned to avoid the shock when the tone comes on, reduce the shock duration to ~0.3 sec. Alternatively, program the computer to deliver a 0.3-sec shock when the mouse successfully avoids, and a longer duration (1 to 3 sec) when it fails to avoid, as an "error-time-out." In both cases, the shock is signaled by the shock-signal light.

    The shock level should be mild so that the animal does not develop a fear of the spout, but returns to it as soon as the warning trial is over. If the shock level is accidentally set too high, the animal will terminate the session by refusing to go back to the spout and provision of supplemental water in its home cage may be necessary. Lower the shock level the next time the animal is used and, if it is still reluctant to drink, turn the shock completely off until it is drinking steadily again. If necessary, increase the reward rate to get the animal to drink again.

32. If a mouse takes too long to return to the spout after a warning trial and if lower shock levels are ineffective in suppressing drinking, program the computer to run the syringe pump while the shock is on so that there is a drop of water waiting for the animal when it returns to the spout—do this only for warning trials that the animal successfully avoided.

33. Use the lowest shock level that elicits reliable avoiding.

34. As soon as an animal has learned to reliably respond to a loud tone, train the animal to respond to lower intensity tones so that it learns to listen.

Determine threshold

Threshold is determined by reducing the intensity of the tone until the performance of the animal falls to chance levels. The rigor of threshold determination depends on the goal of the study. If it is only necessary to show that a particular sound is clearly audible to the animal, then a single descending threshold may be sufficient. Similarly, if the animal's threshold was previously determined and examination is necessary to see if some manipulation has affected it, one descending threshold may be sufficient if it shows no loss of sensitivity. If, on the other hand, the threshold of the animal is being determined for the first time, and requires precise data, then the following procedure may be used.

35. Obtain an approximation of the animal's threshold. Beginning at an intensity ~10-dB lower than the initial training intensity, present a block of trials that includes two

or three warning trials. If the animal avoids on at least two of the warning trials, and its false-positive rate is not high (e.g., <15%), reduce the intensity of the tone by 10 dB and repeat the procedure. Keep lowering the intensity in 10-dB steps until the animal misses two or all three of the warning trials—at this point, present additional trials at that intensity to determine if the animal's threshold has been reached or whether it just needs to listen more carefully. If the animal begins to successfully avoid, decrease the intensity in 5- or 10-dB steps and repeat the procedure.

Be flexible; the goal is to estimate the animal's threshold without it receiving so many shocks that its performance deteriorates.

36. Once threshold has been estimated, increase the intensity to the lowest level that the animal easily detect. Give three to five warning trials at the easily detected intensities and five to eight warning trials at the intensities around and below threshold, then have the computer calculate the performance score as well as the probability level. Reduce the intensity in 5-dB steps until performance falls to chance levels. Then go back up to an intensity that gave an above-threshold score (e.g., 0.75) and make sure the animal is still performing well.

    This is an important step as an animal that does not perform well at the increased intensity may not have been attending to the sound at the lower intensity.

37. Do not give too many warning trials in a row at subthreshold intensities or the animal may stop drinking or stop listening for the tone. If, as a result of subthreshold testing, the false-alarm rate becomes too high (e.g., >20%), increase the intensity by 10 dB or more and keep it there until the false-alarm rate decreases to an acceptable level. Carefully observe the hit rate because an animal given too many undetectable warning trials may stop trying to listen and will miss even higher intensities. If the animal performs satisfactorily at the higher intensity, proceed to accumulate a sufficient number of warning trials (e.g., about eight) at intensities above and below threshold.

    Do not discontinue the shock when testing at subthreshold intensities as the animal will learn that shock does not follow low-intensity sounds and ignore them.

38. Always end a session with the animal performing well. When the session is nearing its end, increase the intensity of the tone to a level the animal should easily detect.

    Good scores at the end of a session indicate that earlier poor scores to low-intensity sounds were not due to lack of motivation or fatigue.

39. Repeat testing until the thresholds obtained in different test sessions are within 3 to 4 dB of each other and not trending up or down.

40. Change to another frequency to train the animal to generalize to other tones.

    Animals seem to generalize better to tones that are close in frequency (e.g., after 8 kHz, go to 4 or 16 kHz).

41. Once thresholds have been obtained for all of the desired frequencies, go back and recheck each one. If the threshold is within the range of the previously obtained thresholds, that frequency can be considered completed. Otherwise, keep testing until a stable threshold emerges.

Calculate threshold

42. Calculate a session average for the different intensities, discarding individual scores that are suspect (e.g., warm-up trials and trials late in the session when the animal was no longer working consistently).

43. Calculate the false-alarm rate separately for each intensity based on the safe trials that occurred when that intensity was being tested. Do not use the session average

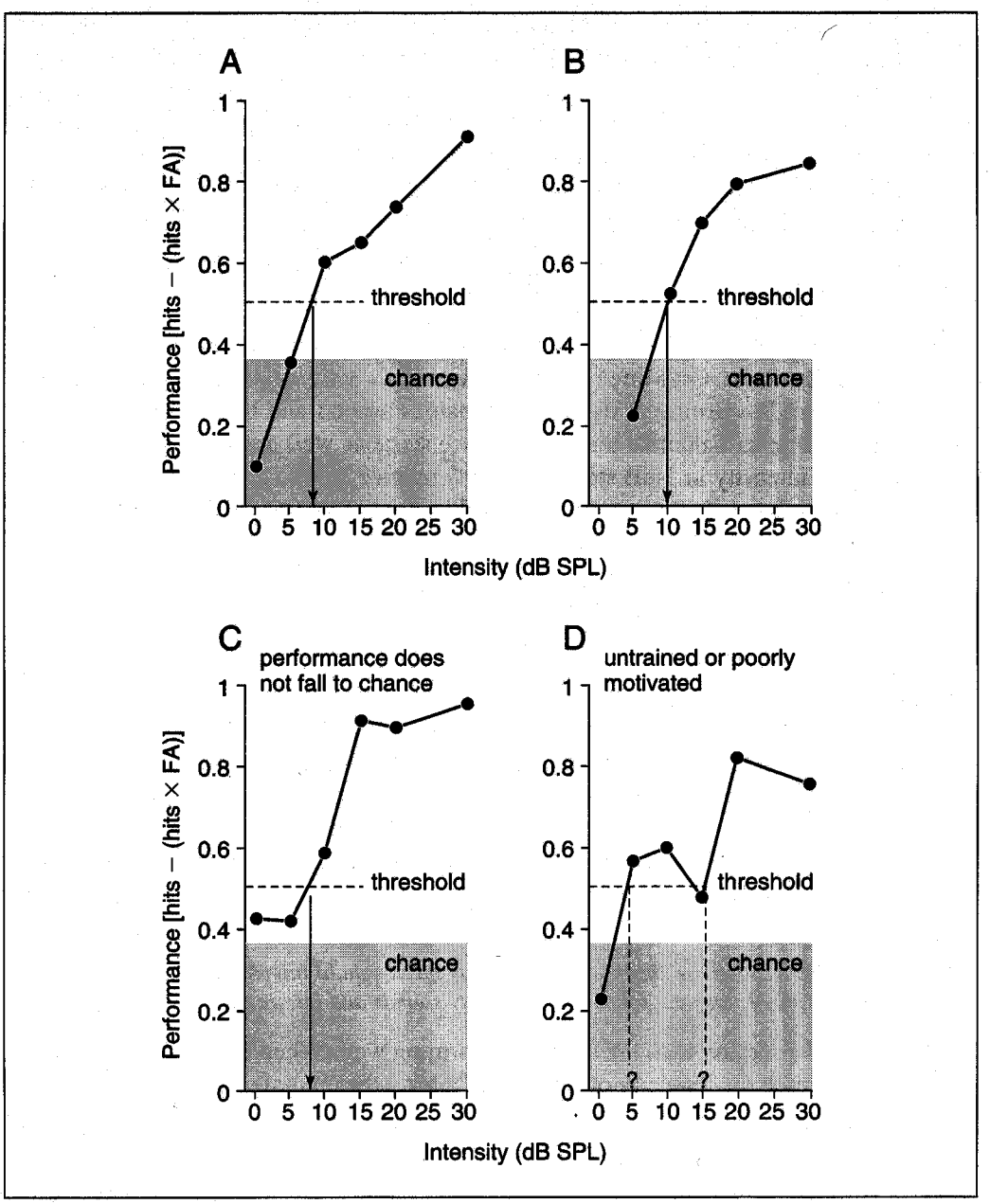

Figure 8.21D.2 Sample psychophysical functions. Panels **A** and **B** illustrate acceptable results whereas panels **C** and **D** indicate methodological problems. The horizontal dashed line indicates the 0.50 level of performance. The shaded area indicates chance performance levels at which the hit and false-alarm rates do not differ significantly. (Modified from Koay et al., 2002.)

of all the safe trial scores to calculate false-alarm rate because false-alarm rates typically vary within a session, being higher for intensities at and below threshold, and lower for suprathreshold intensities.

44. Calculate the hit rate (1–average warning score) and false-alarm rate (1–average safe score) for each intensity and use those values to determine performance, using the formula: performance = hit rate – (hit rate × false-alarm rate).

Threshold is the intensity that yields a performance score of 0.50, which is usually determined by interpolation.

The 0.50 or 50% performance level (in this case, 50% detection corrected for any false alarms) has long been used in psychophysics to define threshold and appears to be more stable than higher or lower performance levels. Moreover, the use of this definition allows easy comparison between thresholds obtained by different procedures.

Analyze and interpret data

A psychophysical function can be generated by plotting an animal's performance for a particular frequency as a function of intensity. This function gives a picture of the performance as well as the 0.50 threshold. Figures 8.21D.2A and 8.21D.2B illustrate psychophysical functions obtained from two mice (F1 offspring of C57BL/6J × C3HeB/FeJ), one that was homozygous for the med^J mutation making it weak and dystonic (Fig. 8.21.D2A), and an unaffected animal from the same F1 background (Fig. 8.21D.2B). Key features of these two functions are (1) the animals performed relatively well at the higher intensities, (2) performance decreased monotonically as threshold was approached, and (3) performance fell to chance at subthreshold intensities. Note that the decrease in performance below the 0.50 threshold is relatively steep.

Figures 8.21D.2C and 8.21D.2D, on the other hand, are hypothetical psychophysical curves that indicate methodological problems. In Figure 8.21D.2C, the psychophysical function declines with intensity, but never falls to chance. Such performance can occur when the animal begins using some other cue to detect warning trials when the tone is no longer audible. It can also occur if the attenuator is no longer reducing the intensity of the tone. In Figure 8.21D.2D, the non-monotonic performance suggests an animal that was either poorly motivated or only partially trained.

Finally, the results obtained from an animal should be immediately compared with those of other animals being tested (if any) as well as with previously published reports. It is best to investigate significant differences in results when it is still possible to re-examine the test equipment, retest an animal, or inspect the animal's ears for signs of abnormality or disease.

COMMENTARY

Background Information

The conditioned suppression method presented here differs in several respects from the original version (e.g., Sidman et al., 1966). The original method used unavoidable shock, had warning trials that lasted up to 60 sec, and compared the number of licks an animal made during the warning trial with the number made during the immediately preceding silent interval. The modifications came about because they offer several advantages. Allowing an animal to avoid the shock increases the number of warning trials that can be given since animals seem to tolerate only a certain number of shocks per session. Decreasing the trial duration from 60 to 2 sec and requiring the animal to break contact for only the last 200 msec of a trial decreases the response cost to the animal (in terms of lost access to water) which, in turn, allows a lower shock level to be used to get the animal to break contact with the spout. Finally, it requires less training time to let the animal make steady contract with the spout than to train it to make discrete licks at a steady rate.

A detailed comparison of the different behavioral methods used to test hearing in mice can be found elsewhere (Heffner and Heffner, 2001). Briefly, the conditioned suppression method is one of the fastest and most reliable techniques for testing the sensory abilities of mammals. One method that may be slightly faster is the shuttlebox, in which an animal is trained to move from one compartment of the box to the other in order to avoid foot shock (Schleidt and Kickert-Magg, 1979). However, a drawback of the shuttlebox is that the head of the animal is not kept in a fixed position, making it difficult to specify the intensity of the sound at the head. Another drawback is the degree of control one has over an animal's false-alarm rate—with the shuttlebox, the false-alarm rate is controlled by the shock level whereas with conditioned suppression, it is controlled by both the shock level and the reward rate, making it easier to keep it from getting too high.

What is an acceptable false-alarm rate depends on the degree of detectability (or discriminability) of the stimulus. For easily detectable stimuli, the false-alarm rate can range from 0% to 15%. However, at threshold levels, the false-alarm rate should be >0% because a zero false-alarm rate may indicate that the shock is too low and the animal is not being vigilant for near-threshold signals. Also,

false-alarm rates at threshold levels may temporarily rise as high as 25% to 30% as the animal begins to receive a number of unwarned shocks. For this reason, the hit rate is always corrected by the false-alarm rate for that intensity; the formula used is: performance = hit rate – (hit rate × false-alarm rate). Another acceptable formula, which gives virtually the same threshold values in well-trained animals, is: performance = hit rate – false-alarm rate. Tests in the authors' laboratory have shown that thresholds calculated using the corrected hit rate did not vary when the shock level was increased, a manipulation that increased both hit and false-alarm rates. Thus, reliable measures of threshold can be obtained over a range of false-alarm rates as long the animals are motivated to listen carefully and the hit rate is corrected for the false-alarm rate.

Using signal detection measures, such as d' or A', is not recommended for two reasons (Heffner and Heffner, 2003). First, the values generated are non-intuitive and cannot easily be compared with the standard 50% detection value. Second, it is sometimes assumed that such measures can compensate for a high false-alarm rate when, in fact, a high rate may indicate that an animal is no longer attending to the sound and is simply guessing, a situation in which signal detection measures do poorly (Green, 1995).

It is theoretically possible to use a tracking procedure to obtain thresholds by having the computer adjust the intensity of the sound up or down based on the response of the animal to the previous intensity. However, such a program would have to provide a way of preventing the animal from being given too many subthreshold tones and thus receiving too many unwarned shocks.

Conditioned suppression can be used not only for detection, but for discrimination tasks as well. For example, it can be used to determine the ability of an animal to discriminate frequency and intensity, as well as the ability to localize sound (Heffner et al., 2001). It can also be used to discriminate between two classes of sounds, such as two types of monkey coo vocalizations and rising vs. falling tones (Harrington et al., 2001). In these cases, safe trials contain one type of signal that can be ignored, and warning trials contain the other type that is followed by shock.

Although conditioned suppression has been used as an equivalence test (Heffner and Harrington, 2002), it is not well-suited for this function. This is because equivalence tests typically involve training an animal to discriminate between two types of sounds and then determining whether it suppresses when a new sound is presented. However, using conditioned suppression, regardless of whether the new sound is followed by shock, the animal has the opportunity to learn how to respond to it as well as to any other new sounds that might be introduced. Although this learning can be delayed by discontinuing the shock for the training sounds, the ability to assess equivalence by this method is limited.

Finally, although measures of neural responses are sometimes used to estimate what an animal can hear, the results bear only a general resemblance to an animal's behavioral audiogram. For example, comparison of the behavioral and auditory brainstem response (ABR; *UNIT 8.21B*) thresholds of mice indicates that the ABR overestimates low-frequency sensitivity and underestimates high-frequency sensitivity (Heffner and Heffner, 2003). Furthermore, although the ABR is often used as a measure of hearing loss in animals due to noise exposure or other insult, it is not known whether the hearing loss as measured by the ABR accurately predicts the behavioral hearing loss. In short, physiological measures of hearing are not valid measures of absolute sensitivity and their validity as measures of hearing loss has never been systematically investigated.

Critical Parameters

There are two important points to keep in mind, (1) dealing with the water needs of mice, and (2) the fact that their hearing range differs from that of humans.

Water needs

The house mouse, *Mus musculus*, has the water metabolism of a desert animal (Bronson, 1984) and a 35- to 40-g adult can maintain its body weight on as little as 1.5 to 2 ml/day. This means that an animal on test should have access only to dry food in its home cage and that the syringe pump delivering the water in the test cage should be able to smoothly dispense as little as $\leq$5 ml/hr.

If animals are not to be tested for a few days, as on a weekend, it is best to give them a measured amount of fluid rather than giving them free access to water and taking it away 24 hr before resuming the test. This is because mice that have had their access to water restricted will increase their water intake when it becomes freely available. As a result, they will not be sufficiently thirsty to work for 2 to 4 days after water restriction is resumed.

Because it can be difficult to accurately give an animal the ~2 ml it will need, one solution is to give it a piece of a fresh, sweet apple, starting with 1.5 g of apple for every 1 ml of water it needs to maintain body weight and adjusting the amount as needed. An alternative source of fluid is a substance such as Transgel, available from Charles River Laboratories, as a source of water for animals while in transit.

Keep in mind that animals with motor problems may have difficulty reaching the food and/or water in their home cage. For example, med^J mice, which have tremors, weak hind limbs, and dystonic postures, have difficulty drinking from a water bottle placed in the grid cover of their solid-bottom cage. In addition to making the water bottle more accessible, their diet can be supplemented with pieces of apple and gelatin placed inside their home cage when they were not on test (Koay et al., 2002). In addition, a cantaloupe/pear juice mixture can be used as a reward in the test cage.

Animals drink less water while on test than they do when given constant access to water, with the result that their body weight typically drops to 80% to 85% of their ad lib weight. This is probably the animals' true normal weight as constant access to food and water is not a normal condition and wild-trapped animals put on an ad lib diet gain weight. It should come as no surprise that animals kept on test for long periods of time will be healthier than those on free feed, as numerous studies have shown that reducing the caloric intake of animals slows signs of aging, decreases the incidence of disease, and increases lifespan (Heffner and Heffner, 1995).

Finally, animals are usually allowed to remain in the test cage until they no longer drink steadily enough to test, by which time an animal should have obtained enough water to maintain a constant body weight. If, however, an animal quits drinking and continues to lose weight, try increasing the reward rate, as mice normally should have no difficulty obtaining sufficient water in one session per day. On the other hand, if an animal obtains so much water that it works poorly the next day, either reduce the reward rate or end the session when the animal has received enough water to maintain a good working weight. Thus, the amount of water an animal should receive in a session is guided by both its body weight and its performance. Keep in mind that immature animals will continue to grow and may increase their working body weight above their initial ad lib weight.

Mouse hearing range

Humans and mice have substantially different, although overlapping, hearing ranges. At a level of 60 dB SPL, the human hearing range extends from 31 Hz to 17.6 kHz (a range of ~9 octaves), with a best sensitivity of about −10 dB at 2 to 4 kHz (Jackson et al., 1999). The 60-dB hearing range of domestic mice, on the other hand, extends from 2 to 88 kHz (a range of ~5.5 octaves), with a best sensitivity of 7 dB at 16 kHz (Table 8.21D.2).

The difference in hearing ranges means one cannot assume that a sound easily audible to humans is similarly audible to mice, an important consideration when choosing sounds to present to mice. For example, because humans have better low-frequency hearing, a 1-kHz tone at 70 dB SPL, which is loud to humans, is completely inaudible to mice. On the other hand, the better high-frequency hearing of mice means that there can be high-frequency components of a sound that are audible to mice, but can be detected by humans only with high-frequency sound measuring equipment.

The better high-frequency hearing of mice, compared with humans, is explained by the need to localize sound. Briefly, mammals with small heads and pinnae need to hear higher frequencies than larger mammals in order to use interaural intensity differences and pinna cues to localize sound. This is because these cues are available only if an animal can hear high enough for its head and pinnae to effectively shadow the sound. Thus, the smaller the functional head size of an animal, the higher it hears. Mammals show even greater variation in low-frequency hearing ability, although the reasons for this variation are not well understood. For a discussion of these points, see Heffner and Heffner (1998, 2003).

Troubleshooting

The following are some common problems encountered with the conditioned suppression protocol presented in this unit along with potential solutions.

Animal does not drink steadily and pauses frequently. The mouse may not be thirsty—check its weight. If the weight is higher than usual, then it may not be working because it is not thirsty. If it is at or below its normal running weight, then its failure to work is due to some other cause. One possibility is that water is not flowing to the spout, perhaps owing to air bubbles in the line.

Animal drinks steadily, but makes intermittent contact by licking. Most mice will make

Table 8.21D.2 Absolute Thresholds of Domestic Mice[a]

Frequency (kHz)	Threshold (dB SPL)
1	92
1.4	78
2	56
3	40
4	30
8	18
16	7
32	22
50	19
64	27
80	43
90	75

[a]Data from Koay et al. (2002).

steady contact with their front paws on the spout. Try adjusting the spout height or its distance from the fence to a more comfortable position for the mouse.

Animal does not hold its head in the proper position. Try adjusting the spout or fence. If this does not work, use a hand-held push-button switch to momentarily stop the water flow whenever the animal has its head in the wrong position.

Animal does not break contact with the waterspout on warning trials. Assuming that the animal can hear the warning sound, try turning the shock up. If using a damp sponge for foot contact, make sure it has been moistened. Some mice, especially those with tremors, make intermittent contact with the spout and often do not feel much of the shock. Try using a shock grid to deliver footshock. If a shock grid is used, be sure to clean it so that it does not get shorted out by feces and urine.

The effect of a single shock level is variable. The animal is likely making intermittent contact such that it does not always get the shock. Try adjusting the spout position or fence. Clean the cage floor with steel wool. Make sure the floor sponge is moist.

False-alarm rate is too high. The shock level is too high or the reward rate is too low.

Animal is slow to return to the waterspout after warning trials. If the shock level is not too high, try giving the animal extra water when it returns to the spout after a successful avoidance.

Performance never falls to chance. The animal may be using another cue (auditory or even visual) to detect warning trials—go in the chamber and carefully look and listen. If it is a detection task, the attenuator may not be reducing the intensity completely. If an amplifier is being used, try using a lower-wattage amplifier or an impedance-matching transformer. In a sound-localization task, an animal may cue in on differences in the quality of the sound emitted by the two loudspeakers and thus never fall to chance—find speakers that have matched output, try randomizing the intensity and quality of the sound from the speakers, or try different speakers.

Thresholds vary within an animal. Detection thresholds should vary by less than ±2 dB. Excessive variation could be due to the sound level varying, inconsistent motivation, or to disease (e.g., middle ear infection or ear mites).

Thresholds vary between animals. Mice tend to have similar thresholds, although some inbred or mutant strains will have a hearing loss. Make sure the animals are equally trained and motivated. If an animal has a zero false-alarm rate at threshold levels, the shock may not be sufficiently high to motivate it to respond. Alternatively, check the animals for head position while drinking, signs of ear infection, and possible exposure to loud sounds.

Anticipated Results

The method of conditioned suppression is a conceptually simple procedure (animals freeze when they hear a sound) that can be used to test the hearing ability of normal mice as well as those with severe neurological defects. As

previously mentioned, it can be used to determine the behavioral ability of an animal to discriminate between any two sounds, or two classes of sounds, as well as to detect a sound. Moreover, the thresholds obtained with this method are reliable, as demonstrated by a test-retest study of absolute thresholds in mice that found that thresholds at frequencies not subject to age-related hearing loss varied by no more than 5 dB (and as little as 0.5 dB) over a 50-week period (Koay et al., 2002). Thus, using this method, one can obtain reliable and valid information about the ability of an animal to detect and discriminate sound.

Time Considerations

Training an animal to make steady contact with the waterspout usually takes an inexperienced researcher using untried equipment five to ten sessions (one ~30-min session per day). Once the procedure has been optimized, subsequent animals may be trained in as little as three sessions.

Training an animal to suppress drinking upon hearing a sound and obtaining a first reliable detection threshold generally takes an additional three to six sessions.

Threshold determination requires obtaining a threshold in one session that is rechecked in two or more subsequent sessions to ensure that it is stable (±2 dB). Thus, each frequency takes three or more sessions.

Once thresholds are known, one to three different frequencies can be rechecked in a single session.

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Key References

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