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Detection of Tinnitus in CBA/CaJ Mice Using the Active Avoidance Shuttle Box Test

Grace Nichols
grace.nichols@uconn.edu

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Committee Chair: Dr. Douglas Oliver

Committee Member: Dr. Charles Giardina

Committee Member: Dr. Monty Escabi

ABSTRACT

Tinnitus is a neurological condition that involves the perception of a sound that is not actually there. Individuals affected with tinnitus describe the condition as a ringing, buzzing, or whooshing sound in their ears. One-third of the population is estimated to have tinnitus, and for many individuals, the condition negatively impacts quality of life. Difficulty falling asleep, trouble with hearing and concentrating, and in some cases, depression, have all been observed to occur with tinnitus.

Tinnitus has been extensively studied in animals, and behavioral tests are the primary method of evaluating the presence of tinnitus in animals. This study evaluates tinnitus induction in CBA/CaJ mice using the Active Avoidance Shuttle Box Test as an assessment for tinnitus. Tinnitus was induced in unanesthetized mice with a unilateral exposure to a 116 dB SPL noise centered at 16 kHz for 1 hour. Half of the mice were exposed to this noise with a 2 kHz-wide bandwidth, while the other half was exposed to the same noise with a ½ octave-wide bandwidth. Behavioral signs of tinnitus were observed in 5 out of the 9 sound-exposed mice. Neither sound exposure appeared to be more effective in inducing tinnitus, however, the bandwidth of the sound exposure may possibly relate to the resulting frequencies of tinnitus observed in mice.

ACKNOWLEDGEMENTS:

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INTRODUCTION:

Tinnitus is a neurological condition that involves the perception of a sound that is not actually there. Individuals affected with tinnitus describe the condition as a ringing, buzzing, or whooshing sound in their ears. Tinnitus is an uncomfortable condition to live with, can negatively impact quality of life, and can even be potentially harmful. Difficulty falling asleep, trouble with hearing and concentrating, and in some cases, depression, have all been observed to occur with tinnitus (Hall et al., 2016). One-third of the population is estimated to have tinnitus, with individuals over 60 years of age and veterans being commonly affected by the condition (Roberts & Eggermont, 2012, Swan et al., 2017). No cure exists for tinnitus, and there is no objective test that can prove that an individual has the condition (Roberts & Eggermont, 2012).

Animal models of tinnitus have been extensively studied in mice, rats, and guinea pigs. Behavioral tests are the primary method used to evaluate the presence of tinnitus in animals. Currently, the most commonly used behavioral test for tinnitus is the gap prepulse inhibition of the acoustic startle reflex (GPIAS) (Galazyuk, et al., 2015, Longenecker, et al., 2011, 2014, 2018). GPIAS relies on a mouse's, or rat's, startle reflex in response to intense, sudden sounds. When short gaps of silence are inserted right before a startling sound, a rodent recognizes the gap, braces for the intense sound to come, and shows a reduced startle response. It is theorized that mice with tinnitus will struggle to recognize these gaps of silence due to their tinnitus "filling in" these short silent periods. Therefore, mice with tinnitus are expected to show a different pattern of startle responses.

While GPIAS is commonly used as a behavioral test for tinnitus, concerns regarding its efficacy still remain. The test relies on the premise that animals with tinnitus will struggle to recognize gaps of silence within a continuous sound. However, there has been supporting evidence that both rats and humans with tinnitus do not have impaired gap-detection abilities (Boyen et al., 2015, Radziwon et al., 2015). It has also been observed that when repeatedly exposed to an intense and startling sound, animals will begin to habituate and show reduced startle responses (Lobarinas et al., 2013, Longenecker et al., 2011, Chen et al., 2013). Such occurrences could impact the GPIAS test's ability to accurately indicate tinnitus in an animal.

A new behavioral paradigm that can potentially be used to assess tinnitus is the Active Avoidance Shuttle Box Test. This test involves training mice to shift to the opposite side of a shuttle box when tones of different frequencies are presented. If a mouse fails to cross over to the opposite side of the box at the presentation of a tone, it will receive a mild electric shock through the floor of the shuttle box. Mice are fully-trained in this task so that they become proficient in avoiding shocks. If tinnitus is induced in a mouse, it is theorized that the mouse will struggle to recognize the warning tones at the frequency of its tinnitus. This will result in the mouse failing to move to the opposite side of the shuttle box, and therefore, receiving a greater proportion of foot shocks at this frequency of the warning tone. Such a deficit in behavioral performance at a specific frequency would indicate tinnitus in a mouse.

The premise for the Active Avoidance (A.A) test was developed from a previous study that involved a similar methodology using lick suppression (Jones & May, 2017). Rats were trained to drink from a water spout only during silent periods. If a broadband

noise was presented, rats would receive a mild shock when they attempted to drink from the water spout. Rats quickly learned to suppress licking during periods of sound presentation, and they were trained until they were proficient in this lick suppression. After induction of tinnitus, it was expected that tinnitus-positive rats would struggle to recognize a warning tone at the frequency of its tinnitus, as its tinnitus would interfere with the perception of the tone. As a result, the lick rates of a rat would increase during the tone presentations that match its tinnitus, while its licking would remain suppressed across all other frequencies of sounds presented.

It is important to note that water deprivation is a part of this behavioral paradigm. Deprivation from food or water can cause additional physiological stress for an animal, which can manifest in changes in behavior that can confound results (Faraco et al., 2014, Brozoski & Bower, 2016). To avoid this problem, the Active Avoidance Shuttle Box Test was adapted.

In the present study, mice were trained in the Active Avoidance test prior to sound exposure. Tinnitus was then induced in unanesthetized mice during a 1-hour, unilateral exposure to a narrowband noise. Mice were divided into two different sound exposure groups. The first group was exposed to a 116 dB SPL noise centered at 16 kHz with a 2 kHz-wide bandwidth. The second group was exposed to a noise of the same intensity and center frequency, but with a ½ octave bandwidth. We determined which of these sound stimulation protocols was most likely to result in signs of tinnitus as measured with the Active Avoidance Shuttle Box Test.

METHODS:

Animal Model

Animals were housed in the University of Connecticut Health Center's animal facility. Care and use of animals aligned with the NIH's Guide for the Care and Use of Laboratory Animals, as well as with the Animal Care and Use Committee at the University of Connecticut Health Center.

Mice of the CBA/CaJ strain will be used for this model. CBA/CaJ mice maintain relatively stable hearing thresholds throughout their lives, and demonstrate age-related hearing loss patterns similar to that of humans (Longenecker et al., 2014). Because of these attributes, CBA/CaJ mice are commonly used in experiments modeling hearing loss and tinnitus (Longenecker et al., 2014, 2011, 2018, Lowe et al., 2015, Wood et al., 2019).

Mice were numbered according to the year that they were acquired (2019) and the chronological order in which they were added to the laboratory's cohort. For example, the fifth mouse acquired in 2019 would be labeled 19-05. Mice were identified by tattoos on their tails. The mice included in this study were 19-06 (F), 19-07 (F), 19-09 (M), 19-16 (M), 19-18 (M), 19-19 (F), 19-22 (F), 19-54 (F), and 19-62 (F). "M" and "F" signify male and female sex.

Hearing Threshold Tests

a. Anesthetic Induction

Mice had their hearing thresholds tested at the age of 2-6 months. Methods used to measure hearing thresholds included the amplitude modulated following response (AMFR) and the auditory brainstem response (ABR).

Mice were anesthetized in one of two ways: in an induction box with 4% isoflurane and 2.0 L/min flow of oxygen for 1-3 minutes, or with an intramuscular injection of 10 mg/mL ketamine and 1.43 mg/mL xylazine in NaCl. After successful induction with ketamine/xylazine, mice were transferred to a heated stage and delivered 0.5 L/min oxygen flow via a nose cone. If isoflurane was used for induction, the mouse was moved to the stage and delivered 1.5-2% isoflurane and 0.5 L/min oxygen via the nose cone. The mouse's reflex was assessed every 20-30 minutes to ensure that the animal was unresponsive.

For the duration of the experiment, mice remained on a heated stage with a rectal probe monitoring and maintaining temperature at 36-38 ℃ (FHC, Bowdoin, ME, USA). A lubricant was applied to the eyes for hydration and 0.3 mL of warm saline was delivered every 30 minutes. The animal's right hind leg was shaved and a thigh-clip monitored vitals throughout the experiment (MouseOx, Starr Life Science Corp, PA, USA). If vitals indicated that a mouse was beginning to wake up, or if a reflex returned, a half-dosage booster anesthetic of 10 mg/mL ketamine was administered. If isoflurane was used for initial induction, the isoflurane flow rate was increased by 0.25-0.50% in lieu of a booster injection.

b. Auditory Brainstem Response (ABR)

All hearing threshold assessments were performed in a sound attenuated chamber (IAC, Bronx, NY, USA). To conduct ABRs and AMFRs, three stainless steel needle electrodes were inserted subcutaneously to record the sound-evoked potentials. The electrodes were inserted following anesthetic induction and the disappearance of the reflex. The ground and reference electrodes were inserted retroauricularly on both the

contralateral and ipsilateral sides of the head. The recording electrode was placed subcutaneously at the vertex (the top and center of the animal's head).

The procedures and analysis of ABRs and AMFRs were reported previously (Burghard et al., 2019).

The sound stimuli for both ABRs and AMFRs were produced by a TDT RZ6 Processor (TDT, Tucker Davis Technology, Alachua, USA). The sound stimuli were delivered to the mouse through a speaker above and in front of the animal's head. TDT BioSig software was used to configure and analyze the ABR recordings.

The ABR recorded evoked potentials in response to 0.2 ms clicks. 512 bipolar clicks were presented at a rate of 21 Hz for each intensity level. Presentation began at an intensity of 0 dB SPL and was increased in 5 dB SPL steps until clear and distinct ABR waveforms were observed. 85 dB SPL was the maximum level of presentation, if needed. TDT BioSig software was used to produce and analyze the ABR recordings.

The output of an ABR recording is a characteristic waveform with seven peaks (Land et al., 2016). The seven peaks each indicate neuronal responses elicited in distinct structures of the auditory pathway of the brain (Laumen et al., 2016). The recorded signals were first bandpass filtered from 300-3000 Hz, and then averaged together to produce an ABR waveform at each intensity level. Sound intensity continued to increase in 5 dB SPL steps until clear and distinct ABR waveforms were observed. The threshold was defined as the halfway point between the intensity where no response was seen, and the next highest intensity where an ABR waveform was indicated.

c. Amplitude Modulated Following Response (AMFR)

In the AMFR hearing threshold assessment, a 1/3 octave narrow band noise with an exp8 filter was presented at each of the following center frequencies: 8, 12, 16, 24, and 32 kHz. The amplitude of the sound's waveform is normally modulated at a rate in the range of 20-500 Hz. The specific modulation frequency used in these experiments was 42.9 Hz. A modulation frequency of 42.9 Hz is slow enough to ensure that the majority of auditory structures will be stimulated and generate a response (Fitzpatrick et al., 2002).

Each frequency was initially presented at an intensity level at least 15-20 dB SPL above a mouse's average threshold for that frequency, and then in decreasing 5 dB SPL steps until the threshold was identified. The recorded response indicated how well the mouse's brain followed the modulation of the sound, as well as how intense a sound needed to be in order for the brain to register its presence (Griffiths et al., 1991). Raw recordings of evoked potentials were grouped together in sets of 8 epochs, which are considered a "block." 1 epoch is equivalent to 10 cycles, or in other words, a minimum of 250 ms.

The AMFR software and analysis was run through our laboratory's AMFR program in MATLAB. The coherence and coherence strength values of each block were individually calculated to determine whether a response was elicited from the brain. The coherence value indicates how closely the brain's evoked potential follows along with the modulation rate of the stimulus (Onslow et al., 2011). Coherence was calculated in the MATLAB program with the "mcohere" function. Coherence strength was calculated with the following equation:

$$CS = (COH\text{-}N_{noise})/SD_{noise}$$

Where CS is equal to the coherence strength, N_{noise} and SD_{noise} correspond to the mean and standard deviation of the noise floor, and COH represents the magnitude squared COH value.

A coherence value greater than or equal to 0.25, and a strength value greater than or equal to 3.00, were both needed for a block to be considered a successful "pass." A pass indicated that a mouse was able hear the presented sound stimulus. Once 5 consecutive blocks passed, the program moved on to present the same frequency of sound at an intensity level 5 dB SPL lower.

The program continued to present the same sound stimuli in decreasing 5 dB SPL steps until the brain was no longer observed to give sufficient responses. If 5 consecutive passing blocks did not occur, and 350 epochs had passed (approximately 43 blocks), the mouse "failed" to hear the sound at this intensity, and the program stopped running. The threshold was determined by taking the average of the lowest intensity level that successfully passed, and the next lowest intensity level that failed. For example, if a presentation of the frequency16 kHz was observed to pass at 10 dB SPL, but failed at 5 dB SPL, then the threshold for this frequency would be 7.5 dB SPL.

Each of the five different frequencies (8, 12, 16, 24, and 32 kHz) were independently tested to find the respective thresholds.

Active Avoidance Training

After baseline hearing thresholds were evaluated and a mouse was confirmed to have normal hearing capabilities, it began training in a learned avoidance task. The

behavioral task used was the Active Avoidance Shuttle Box Test. This behavioral training served as the primary indicator of whether an animal had developed tinnitus.

The shuttle box was obtained from Panlab, and the Active Avoidance software was developed by Dr. Bradford May (Johns Hopkins University School of Medicine) and run through MATLAB.

In Active Avoidance (A.A.) training, a mouse was placed in one of two shuttle boxes that were connected via a small doorway. The floors of each chamber were made up of rows of metal bars, capable of delivering small electric shocks (0.1 mA). During a training session, a mouse was first given a 5-minute habituation period during which it was allowed to explore the chamber. After the habituation period ended, different tones were presented to the mouse at random time intervals ranging from 10-30 seconds. Each tone was randomly centered at either 11.2, 16, 22.6, or 32 kHz, with the frequency randomly roving a ¼ octave above or below the center frequency. These tones were presented at an intensity of 70 dB SPL, which also randomly roved by 2 dB SPL.

Once a tone was presented, the mouse had 5 seconds to move through the doorway to the opposite side of the shuttle box. If the mouse did not move within these 5 seconds, a 0.1 mA shock was delivered through the floor grid while the tone continued to play. Both the shock and the tone continued until the animal moved to the opposite side of the shuttle box. Once the animal successfully crossed to the opposite side, both the tone and the shock stopped. If 15 seconds had passed since a shock began, and a mouse still failed to shift to the opposite side, both the tone and the shock stopped and a new trial would begin. The training session lasted a total of 45 minutes, including the 5-minute habituation period. Mice were only trained in A.A. once per day.

An “avoidance response” occurred when the mouse moved to the opposite side of the box within the first 5 seconds of the tone’s presentation. This signified that the mouse recognized the tone as a conditioned stimulus, and shifted to the opposite side of the box to avoid being shocked (a conditioned response). An “escape response” occurred when a tone was presented, but the mouse did not move within 5 seconds and was delivered a shock. As a result, the mouse would run to the opposite side of the shuttle box in order to escape the shock. The goal of the A.A. behavioral task is to train mice to successfully recognize tones of all frequencies (conditioned stimuli) as signals to move to the opposite side of the shuttle box (a conditioned response). A mouse's performance was evaluated by its percentage of successful “avoidance responses” for each frequency.

Animals were trained in the A.A. task once per day, 5 days per week, until they mastered the behavioral task. If a mouse was showing signs of distress, it was given several days break from the behavioral training. A mouse was considered fully trained in A.A. once it displayed 5 consecutive days of high avoidance responses. This was defined as having approximately >80% avoidance for each of the four center frequencies that were presented (11.2, 16, 22.6, and 32 kHz). Such results indicated that a mouse recognized tones of all frequencies as conditioned stimuli, and that the mouse successfully moved to the opposite side of the shuttle box >80% of the time. If 10 days of training had passed, and a mouse was not showing improvement, it stopped A.A. training and was removed from the cohort.

Once a mouse completed A.A. training, it was ready to be sound-exposed in order to induce tinnitus. The A.A. task was enlisted following sound exposure in order to behaviorally determine whether tinnitus had been induced.

Sound Exposure

Tinnitus induction was carried out through exposing awake mice to an intense and long-duration sound. Mice were unilaterally exposed to a 116 dB SPL narrow band noise centered at 16 kHz with a bandwidth of 2 kHz, or a half-octave bandwidth, for one hour. Exposure to this acoustic stimulus has been previously observed to induce tinnitus in mice. However, in many cases the mice were anesthetized during exposure, and a different behavioral paradigm was used for tinnitus detection (Longenecker et al., 2011, 2014, 2016, Turner et al., 2012).

Mice were split equally into two groups, one of which will be exposed to the stimulus with a 2 kHz bandwidth, and being exposed to the stimulus with a half-octave bandwidth. A 2 kHz wide bandwidth around 16 kHz ranges from 15-17 kHz, while a half-octave wide bandwidth ranges from 13.454-19.207 kHz.

To unilaterally expose mice, the right ear was plugged with a foam earplug that was cut down to size. Effective earplugs have been shown to reduce sound levels significantly below 116 dB SPL stimuli, which will protect an animal's ear from hearing damage and potentially prevent tinnitus induction on that side (Turner et al., 2006). To insert earplugs, the mice were anesthetized with 4% isoflurane and 2.0 L/minute of oxygen in an induction box for 2-4 minutes. The mouse was then removed and an earplug (already cut down to size) was rolled up, inserted snugly into the animal's right ear, and was allowed to expand. Liquid bandage was used to temporarily bind the outer segment of the earplug to the pinna. This created a seal, and also prevented the mouse from dislodging the earplug during the sound exposure. Animals typically awaken in 1-2 minutes following this anesthetic protocol.

Mice were sound exposed in the anechoic chamber of UConn Health in Farmington, CT. The anechoic chamber provided an open field environment, which minimized any echoes or reverberations. Minimizing reflections of sound provided a controlled environment, meaning that resulting tinnitus, or potential hearing damage, could be presumed to be due to the sound stimuli alone.

Sound was delivered to mice through two horn speakers that face each other. The horns were the H290 model from Eminence, and the speaker drivers were the N151M-8Ω model, also from Eminence. In between the two speakers were two mice, housed in a wire cage and separated by a wire mesh divider. The ample holes in the cage and divider allowed for sound to freely pass through. To ensure that the sound passed through the cage unaltered, the speaker output was checked at different locations inside of the cage with a ¼" microphone (Type 4135, Bruel & Kjaer, Naerum, Denmark). Sound intensity levels and speaker functionality were checked before each mouse pair was sound exposed.

Tinnitus Induction

After animals were sound exposed, their earplugs were removed and they were put back with their respective cage mates. Tinnitus, if successfully induced, developed over the following 8 weeks. While there is not a consensus on a standard timeframe for tinnitus development following such a sound exposure, communication with colleagues suggested that 8 weeks of induction time improved the likelihood of behavioral detection of tinnitus (Dr. Bradford May, Johns Hopkins University School of Medicine).

Post-Sound Exposure Hearing Threshold Tests

A second hearing test was performed following the sound exposure to determine whether a mouse's hearing capabilities were conserved, and if the earplug was successful in blocking the sound. This threshold test took place at least three weeks following the sound exposure. By this time, any temporary threshold shifts, which typically occur following such an intense sound exposure, should disappear. This hearing threshold test served as an indicator of whether any substantial damage to the animal's hearing has occurred.

The same anesthetic protocol and methodology described in "Hearing Thresholds," was used. An additional step taken was after overall thresholds were established (via click ABR, AMFR, or both): the unexposed ear was plugged again, and an AMFR was conducted on the sound-exposed ear alone. For this experiment, the reference electrode was moved to the side of the open ear in order to ensure that recorded signals were coming from the ear receiving the sound stimuli. This unilateral hearing threshold test more clearly demonstrated hearing damage present in the exposed ear, as well as how much the plugged/unexposed ear may be compensating for this deficit.

The post-sound exposure hearing threshold test indicated whether a mouse was able to resume behavioral testing in the Active Avoidance (A.A.) task. Reliability of the A.A. results depended on an animal having normal hearing capabilities. If an animal with significant hearing damage were to resume the A.A. task, then deficits in its performance that were due to hearing loss could be misidentified as tinnitus (a false positive). If it became apparent that the sound exposure caused significant damage to a mouse's hearing, then the mouse did not resume behavioral testing.

Behavioral Determination of Tinnitus Induction

After 8 weeks passed since the initial sound exposure, and no significantly elevated thresholds were seen in the second hearing threshold test, mice resumed testing in the Active Avoidance (A.A.) task. Behavioral results produced from the A.A. test indicated whether an animal had developed tinnitus.

The duration of each A.A. session remained the same: 45 minutes total, starting with a 5-minute habituation period and then 40 minutes of testing. The reinforcement rate of the shock was decreased from 100% to 50%. Mice also underwent testing every other day, instead of consecutive days in a row. This new training schedule was enlisted to prevent exhaustion and learned helpless in mice, which can occur when a potentially stressful behavioral task is carried out for a long period of time (Maier, S.F., 2011, Chourbaji et al., 2005).

The sound intensity of the A.A. tones were decreased from 70dB SPL to 60dB SPL, with the intensity still randomly roving above and below 60 dB SPL by 2 dB SPL. The hearing threshold tests done post-sound exposure indicated whether mice were able to hear a sound at 60 dB SPL. The goal of decreasing sound intensity was to make it more difficult for an animal to discriminate between the warning tones and their own tinnitus. If, hypothetically, an intensity of 70 dB SPL was at least 30 dB SL (sensation level), the mouse would potentially hear and successfully respond to all of the 70 dB SPL warning tones. The mouse may not demonstrate behavioral signs of tinnitus, even if it does have tinnitus, resulting in a false negative. To avoid this scenario, the sound intensity was lowered to 60 dB SPL, making it more likely for a mouse to show deficits

in performance due to its tinnitus. Possible behavioral deficits due to hearing loss were out-ruled by the post-exposure hearing threshold test.

If a mouse had tinnitus, then when a tone at the same frequency of its tinnitus was played in the A.A. task, the mouse would have trouble recognizing the tone because its tinnitus "masked" the sound. The animal would fail to move to the opposite side of the shuttle box, receive a shock, and its avoidance percentage for that particular frequency would drop. Frequencies of sound that are outside of the animal's tinnitus would not be impacted. An animal was only expected to show behavioral deficits at the frequencies of its tinnitus.

Mice completed a total of 5 A.A. sessions, which was sufficient to determine whether behavioral signs of tinnitus were present. Behavioral signs of tinnitus were indicated when low performance at a specific frequency (or frequencies) of a tone was seen consistently across multiple sessions. Our laboratory's A.A. software allowed for a mouse's performance on individual days, as well as across multiple sessions, to be analyzed. From these analyses, consistent low performance at particular frequencies could be seen.

Investigating Neurological Characteristics of Tinnitus

Once the sound-exposed animals with tinnitus were identified, they underwent further electrophysiological testing. Through comparison of results between sound exposed mice with tinnitus, sound exposed mice without tinnitus, and mice that have not been sound exposed, neurological behavior and trends that could be characteristic of tinnitus were investigated.

Due to time constraints, as well as the halting in research activities in light of COVID-19, the electrophysiological analysis of tinnitus is not complete at this time.

Euthanasia

Once all testing was completed, mice were humanely sacrificed according to the laboratory's protocol. Two methods of euthanasia were used: cervical dislocation while under anesthesia, followed by decapitation.

RESULTS:

The goal of this experiment was to investigate whether a 1-hour duration 116 dB SPL noise centered at 16 kHz with a ½-octave bandwidth, or a 2 kHz-wide bandwidth, was more likely to induce tinnitus in mice. Mice were unilaterally exposed to either the noise with a ½-octave bandwidth, or a 2 kHz-wide bandwidth, while awake and without anesthesia. An earplug was inserted into the right ear of each mouse prior to sound exposure.

AMFR and ABR tests confirmed whether hearing thresholds were conserved following sound exposure. Development of tinnitus after sound exposure was assessed with the Learned Active Avoidance behavioral test. It was predicted that mice exposed to the 116 dB SPL noise centered at 16 kHz with a 2 kHz-wide bandwidth would be more likely to develop tinnitus, as the sound was concentrated across a narrower band of frequencies.

Experimental Groups

10 CBA/CaJ mice were initially included in this experiment. Unfortunately, one mouse died unexpectedly before completing the study. The following results are that of the remaining 9 mice.

The mice were separated into two sound exposure groups. One group was exposed to a 116 dB SPL noise centered at 16 kHz with a ½-octave wide bandwidth for 1 hour, while the other group was exposed to a noise with the same intensity level and center frequency, but with a 2-kHz wide bandwidth. Mice 19-06, 19-16, 19-18, and 19-54 were exposed to the sound with the 2-kHz wide bandwidth. Mice 19-07, 19-09, 19-19, 19-22, and 19-62 were exposed to the sound with the ½-octave wide bandwidth.

Tinnitus Assessment Methods

All mice were fully trained in the Learned Active Avoidance (A.A.) task before undergoing sound exposure. Mice were considered "fully trained" once the average of their avoidance percentages across 5 consecutive training sessions was approximately ≥80% across all frequencies. After this was achieved, mice were exposed to the 16 kHz, 116 dB SPL noise for 1 hour with either a 2 kHz-wide bandwidth, or a ½-octave bandwidth.

Eight weeks following sound exposure, mice resumed testing in A.A. The behavioral performance of each mouse from before (PRE, in blue) and after (POST, in red) sound exposure are displayed in the line graphs (Figures 1.2-1.5 and 2.2-2.6). The PRE and POST datasets are each an average of 5 separate A.A. sessions. The PRE dataset

demonstrates a mouse's high performance when fully trained in the A.A. task. The POST dataset shows the behavioral performance following sound exposure and potential tinnitus induction. Labeled points on the POST line graph are frequencies for which a mouse's mean avoidance percentage was significantly below the mean of the overall POST avoidance percentage ($p < 0.05$).

The same POST A.A. data was also plotted in the Surface Profiles, which appear as the yellow/blue heat maps (Figures 1.2-1.5 and 2.2-2.6). Surface Profiles demonstrated a mouse's performance during each of the 5 A.A. sessions. Each red point indicates a frequency for which a mouse's mean avoidance percentage was significantly below the mean avoidance percentage for that A.A. session ($p < 0.05$). The line graphs of A.A. performance and corresponding Surface Profiles can be seen below:

For the mice whose audiograms and A.A. performance did not indicate substantial hearing loss, deficits in A.A. performance were analyzed for potential tinnitus. The identification of tinnitus in a mouse was two-fold. First, there must have been a significant decrease in the mean avoidance at a single frequency, or multiple frequencies ($p < 0.05$), among the POST A.A. data. To test this, a t-test assuming equal variances was used to compare the mean of the overall POST avoidance percentage to the mean POST avoidance percentage for a specific frequency of tone. This analysis revealed whether mice were performing significantly worse at a specific frequency of the warning tone, while still demonstrating high performance at all other frequencies of the warning tones. The frequencies that were demonstrated to be statistically significant are labeled in the line graphs (Figures 1.2-1.5 and 2.2-2.6).

In addition to this parameter, significantly lower performance at this same frequency must have been observed in at least 2 out of the 5 POST A.A. sessions. The Surface Profiles show each of the 5 A.A. sessions plotted against the frequencies of the tone presentations (Figures 1.2-1.5 and 2.2-2.6). The plots are heat maps, with blue areas identifying frequencies with lower avoidance percentages, and red points indicating a statistically significant decrease in the avoidance percentage from the mean avoidance percentage of that session (alpha = 0.05, $p < 0.05$).

If a significant decrease from the mean avoidance percentage was present at the same frequency across multiple sessions, then the red dots would line up with one another and the blue areas would bleed together. For tinnitus to be indicated at a frequency, first the avoidance percentage at that frequency must be deemed significant by the t-test, and second, the avoidance percentage at this frequency should be significantly decreased in at least 2 out of the 5 A.A. sessions.

Tinnitus Assessment of Mice Exposed to Noise with 2 kHz-wide Bandwidth

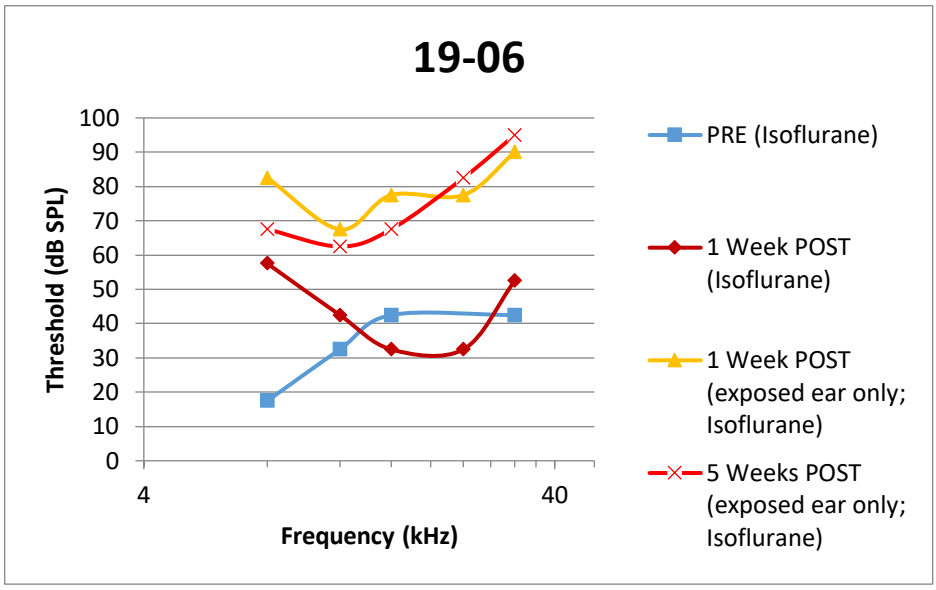
Mice 19-06, 19-16, 19-18, and 19-54 were exposed to the 1-hour duration, 116 dB SPL noise centered at 16 kHz with a 2 kHz-wide bandwidth. The ABR thresholds from before (PRE) and after (POST) sound exposure can be seen below:

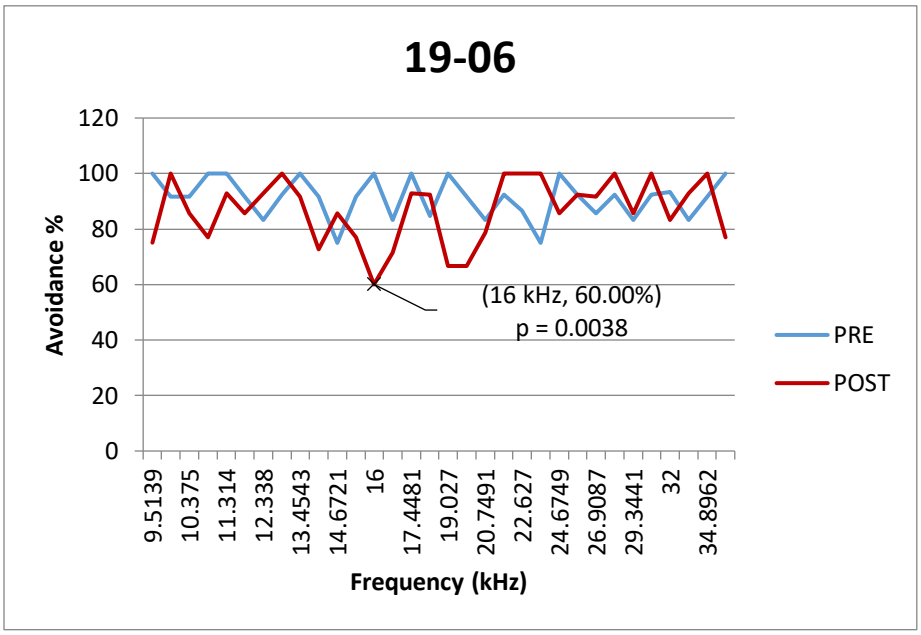
	19-06	*19-16*	*19-18*	*19-54*
PRE (dB SPL)	37.5	27.5	22.5	12.5
POST (dB SPL)	27.5	62.5	22.5	27.5

***Figure 1.1**: ABR thresholds of mice exposed to the 116 dB SPL noise centered at 16 kHz with a 2 kHz-wide bandwidth from before sound exposure (PRE) and after sound exposure (POST).*

Almost all mice in this group appeared to recover their ABR thresholds following sound-exposure (Figure 1.1). However, mouse 19-16's ABR threshold nearly doubled following sound exposure. This indicates an overall elevation of hearing thresholds, and possible damage to hearing in mouse 19-16.

The behavioral results from the Active Avoidance test, along with hearing thresholds from the AMFR test, are shown below for mice 19-06, 19-16, 19-18, and 19-54:





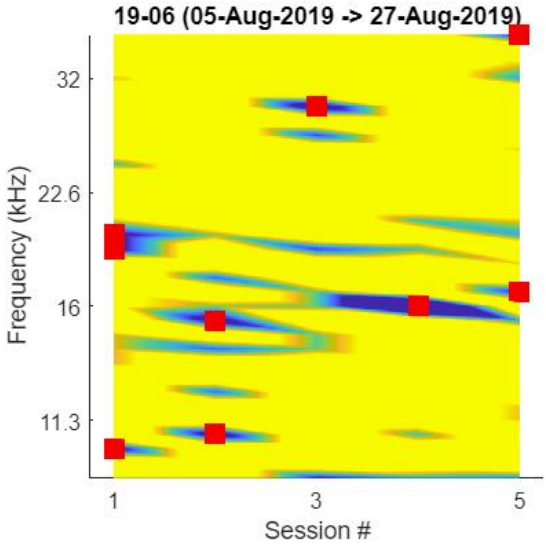
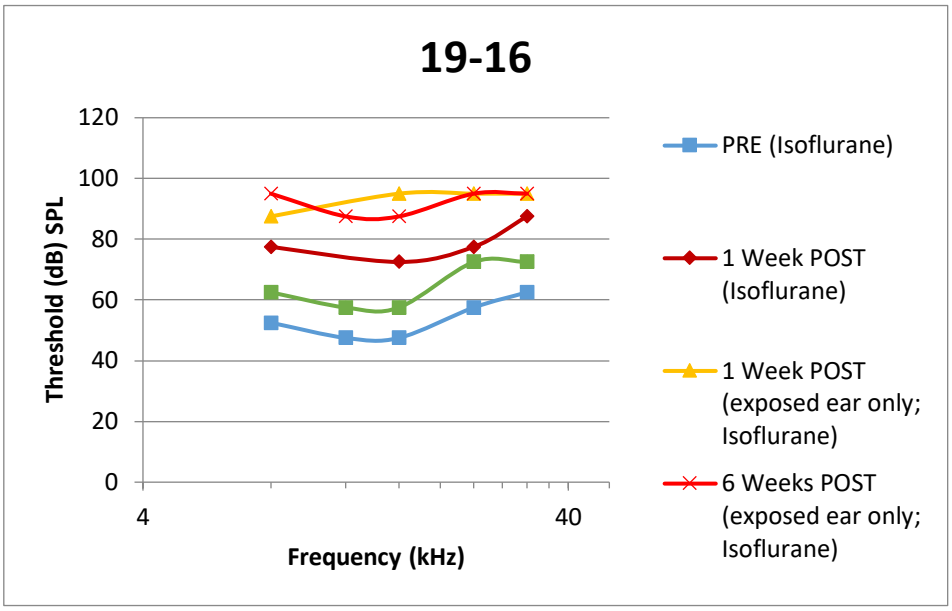
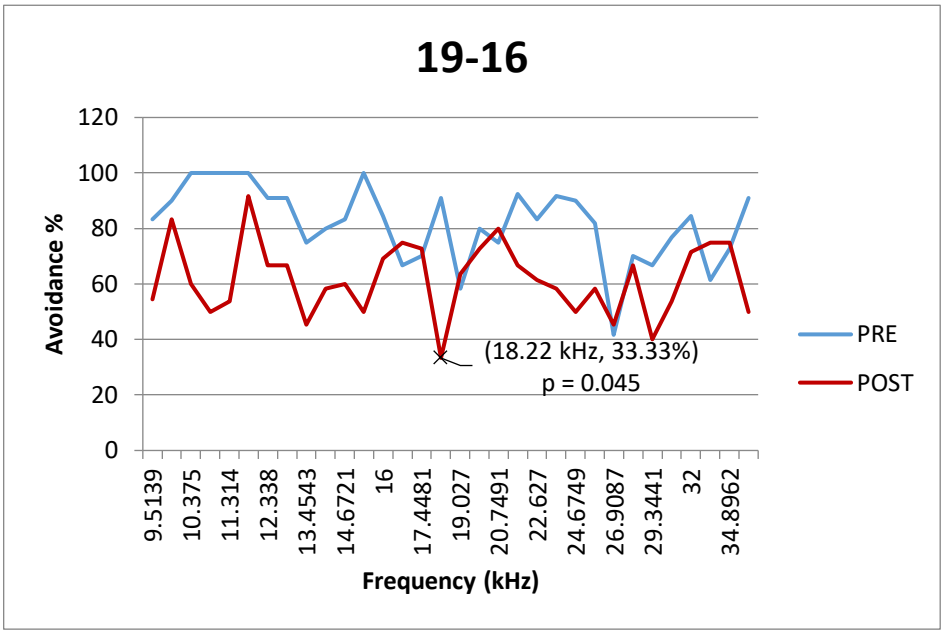


Figure 1.2: *The audiogram and A.A. behavioral results for mouse 19-06. Tinnitus was not indicated by behavioral results.*





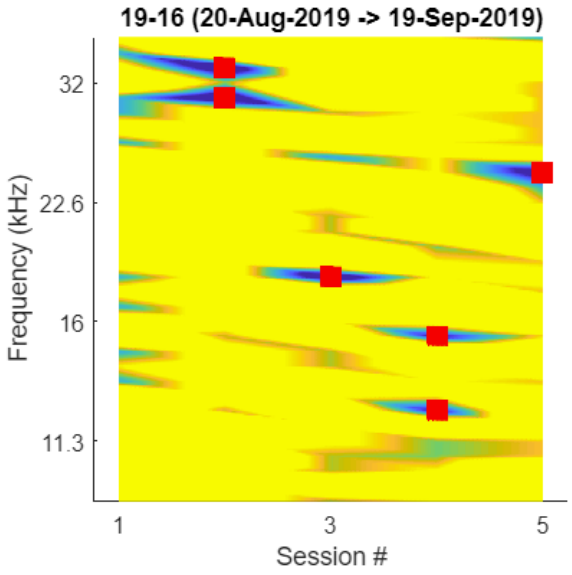
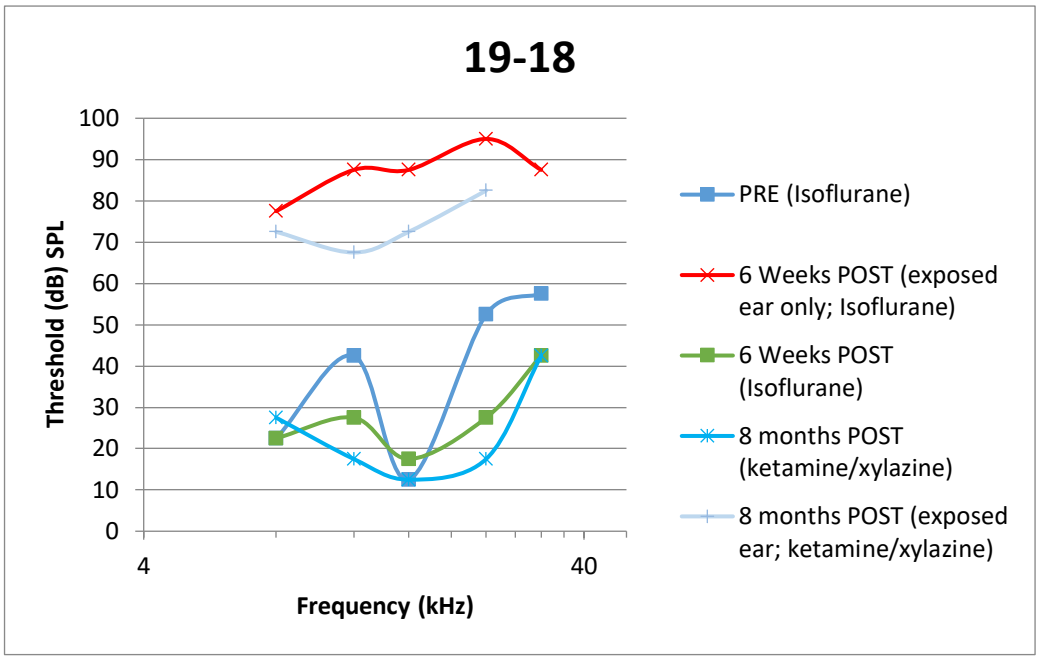
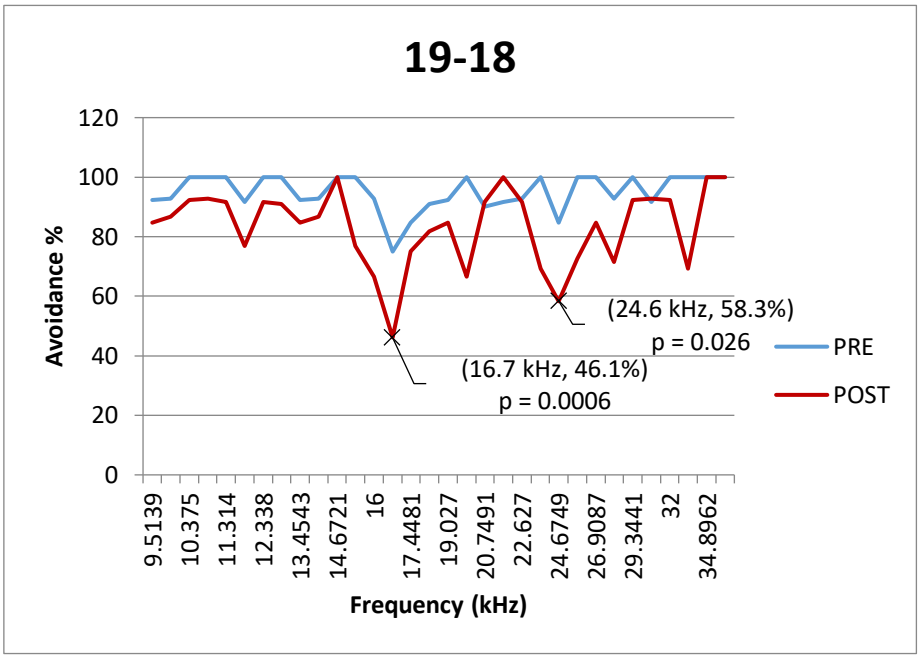


Figure 1.3: *The audiogram and A.A. behavioral results for mouse 19-16. Tinnitus could not be assessed due to elevation in hearing thresholds.*





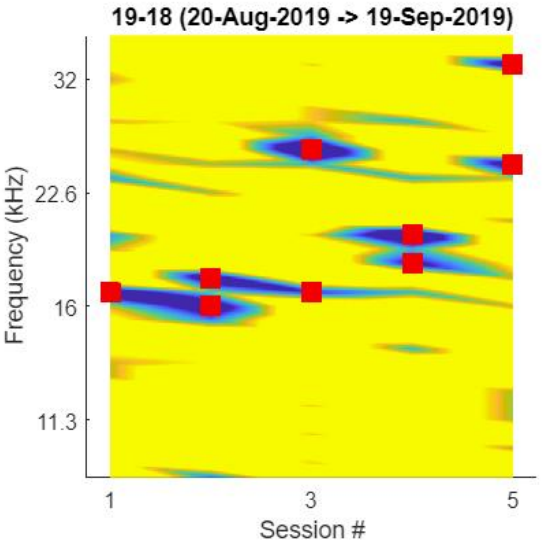
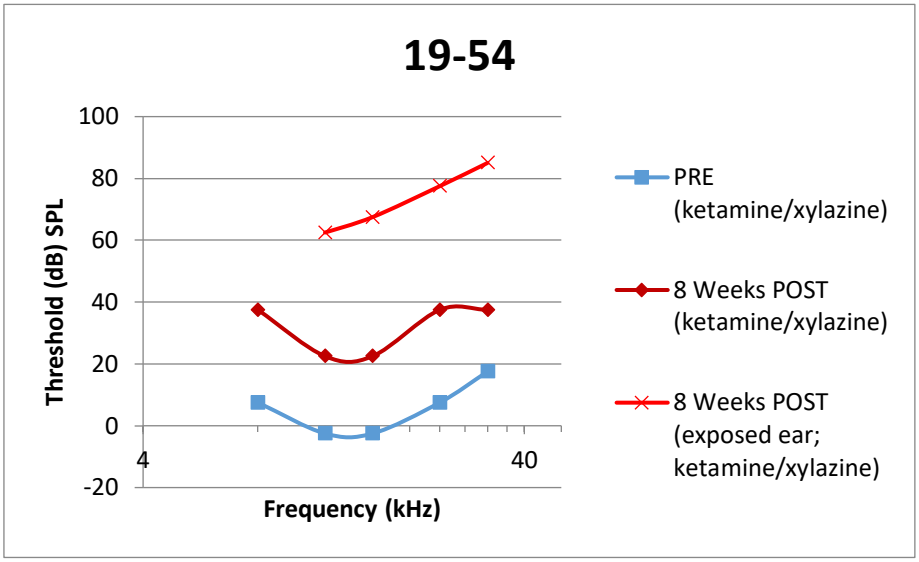
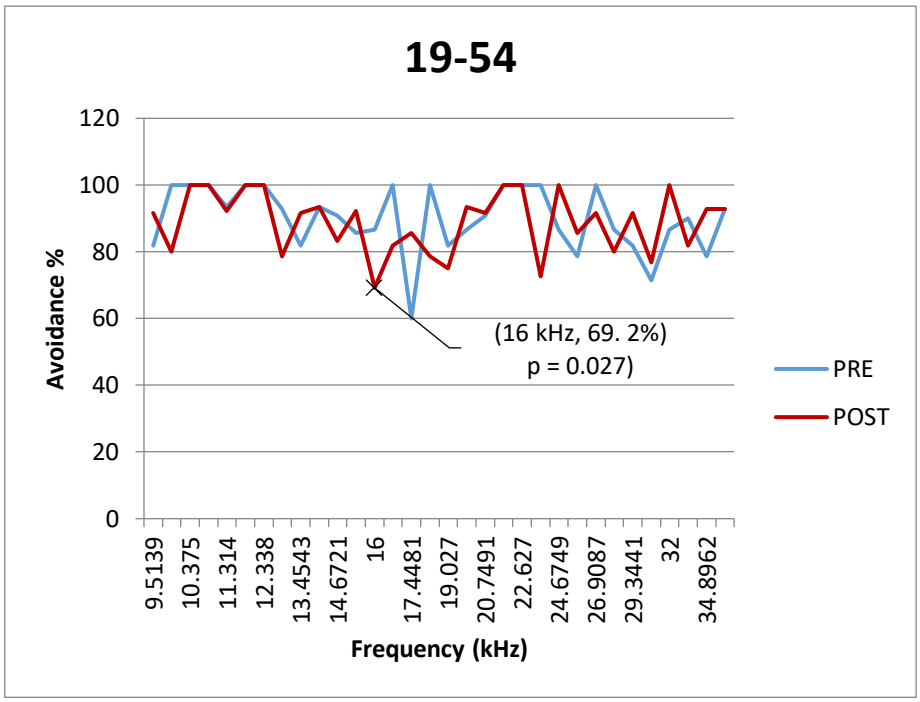


Figure 1.4: *The audiogram and A.A. behavioral results for mouse 19-18. Tinnitus was indicated at 16.7 kHz.*





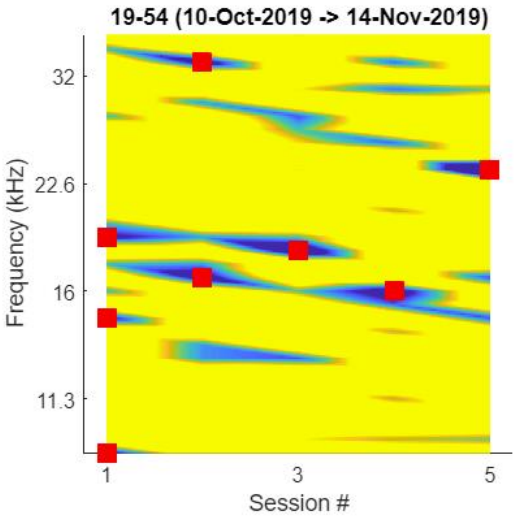


Figure 1.5: *The audiogram and A.A. behavioral results for mouse 19-54. Tinnitus was not indicated by behavioral results.*

Mouse 19-06 was not classified as having tinnitus by the study's parameters. Mouse 19-06's hearing thresholds (ABR and AMFR) nearly recovered following sound exposure, indicating that its behavioral performance in A.A. was not impacted by deficits in hearing (Figures 1.1 and 1.2). Although mouse 19-06 demonstrated a significant decrease in avoidance percentage at 16 kHz ($p = 0.0038$), the Surface Profile did not show behavioral deficits at this frequency across multiple sessions (Figure 1.2). Therefore, mouse 19-06 was not classified as having tinnitus by the study's paradigm.

Mouse 19-16 could not be evaluated for tinnitus. Elevation in hearing thresholds were evident in the ABR following sound exposure (Figure 1.1). Evidence of elevated thresholds were present in the AMFR before sound exposure, as well (Figure 1.3). Because behavioral deficits cannot be discerned from deficits due to impaired hearing capabilities, the A.A. results could not be analyzed for tinnitus.

Mouse 19-18 was found to have tinnitus at 16.7 kHz. Both ABR and AMFR thresholds returned to pre-sound exposure levels, indicating that no overall damage to hearing took place (Figures 1.1 and 1.4). A significant decline in avoidance percentage was found at 16.7 kHz and 24.6 kHz, however, behavioral deficits at the same frequency across multiple A.A. sessions was only seen for 16.7 kHz (Figure 1.4). It is for this reason that mouse 19-18 was observed to have tinnitus at 16.7 kHz.

Mouse 19-54 was not found to have tinnitus based upon the study's parameters. Although the mouse's hearing thresholds appeared to be slightly elevated through the AMFR and ABR tests, the mouse's performance in the A.A. test indicated that it could adequately hear the 60 dB SPL tones that were presented (Figure 1.1 and 1.5). With its avoidance percentages being >80% at almost all frequencies following sound exposure,

mouse 19-54 performed almost as well as did before sound exposure (Figure 1.5). Although the frequency of 16 kHz was found to be statistically significant, the Surface Profile indicated that deficits at this frequency were not present over multiple A.A. sessions.

Based on the study's two-fold paradigm, only one out of the four mice exposed to the 116 dB SPL noise centered at 16 kHz with a 2 kHz-wide bandwidth was classified as having tinnitus. The frequency of tinnitus for this mouse (19-18) was 16.7 kHz.

Tinnitus Assessment of Mice Exposed to Noise with ½ Octave Bandwidth

Mice 19-07, 19-09, 19-19, 19-22, and 19-62 were exposed to the 1-hour long, 116 dB SPL noise centered at 16 kHz with a ½-octave bandwidth. The ABR thresholds from before sound exposure (PRE) and after sound exposure (POST) can be seen below:

	19-07	*19-09*	*19-19*	*19-22*	*19-62*
PRE (dB SPL)	22.5	27.5	22.5	22.5	7.5
POST (dB SPL)	32.5	-	47.5	32.5	12.5

Figure 2.1: *ABR thresholds of mice exposed to the 116 dB SPL noise centered at 16 kHz with a ½ octave bandwidth from before sound exposure (PRE) and after sound exposure (POST). POST ABR threshold for 19-09 was conducted with the wrong configuration file – AMFR results will be used to evaluate 19-09's hearing thresholds.*

Mice 19-07, 19-22, and 19-62 were all found to have ABR thresholds following sound exposure that were similar to their initial, pre-sound exposure thresholds (Figure 2.1). Mouse 19-19's ABR threshold doubled following sound exposure, indicating an elevation of hearing thresholds and potential damage to hearing (Figure 2.1). Mouse 19-09 had a post-sound exposure ABR conducted, however, the wrong configuration file

was used when running the software. AMFR thresholds will be the primary indicator of 19-09's hearing capabilities.

The behavioral results from the Active Avoidance task and the hearing thresholds from the AMFR tests are shown below for mice exposed to the 116 dB SPL noise centered at 16 kHz with a ½-octave bandwidth:

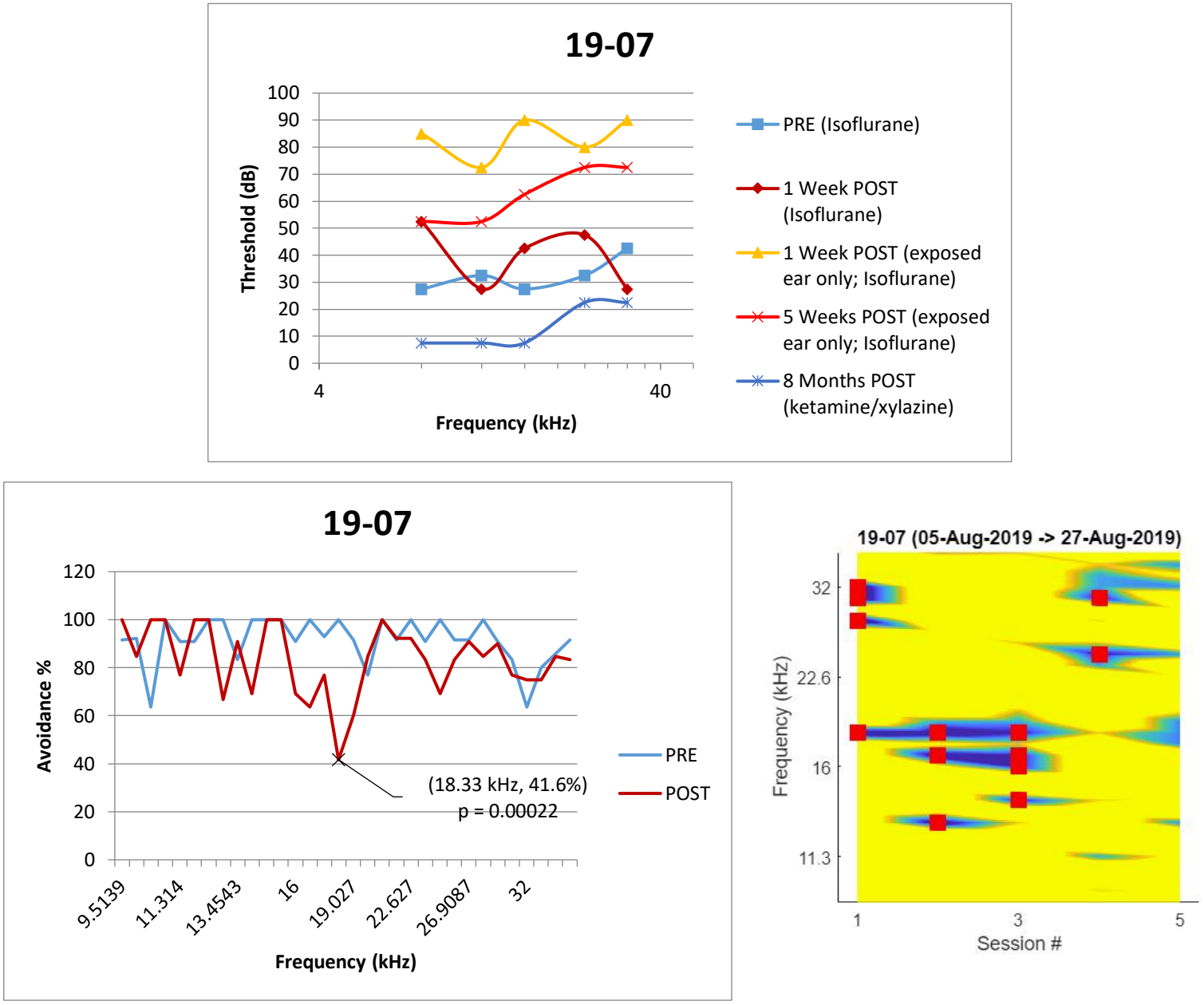
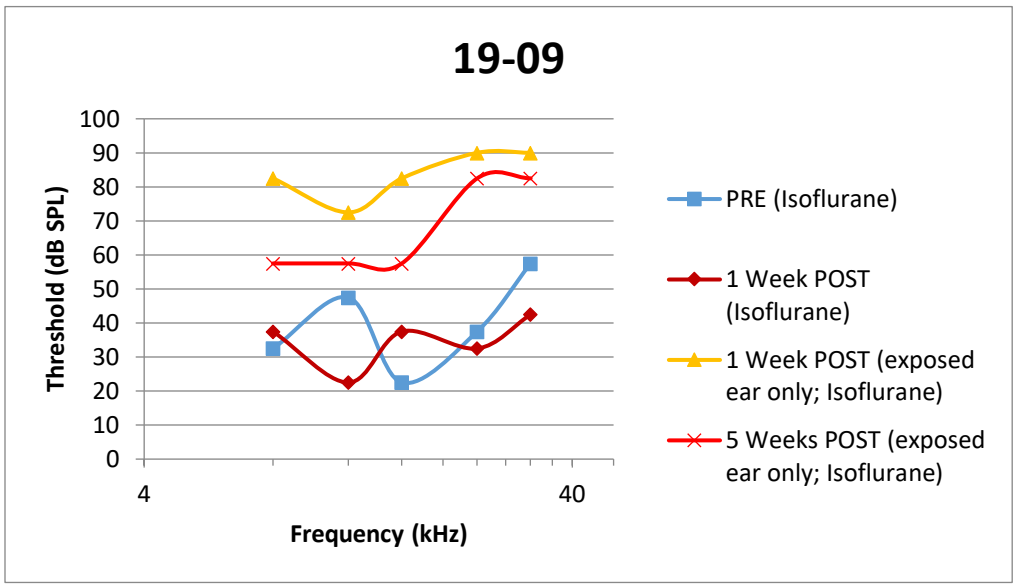
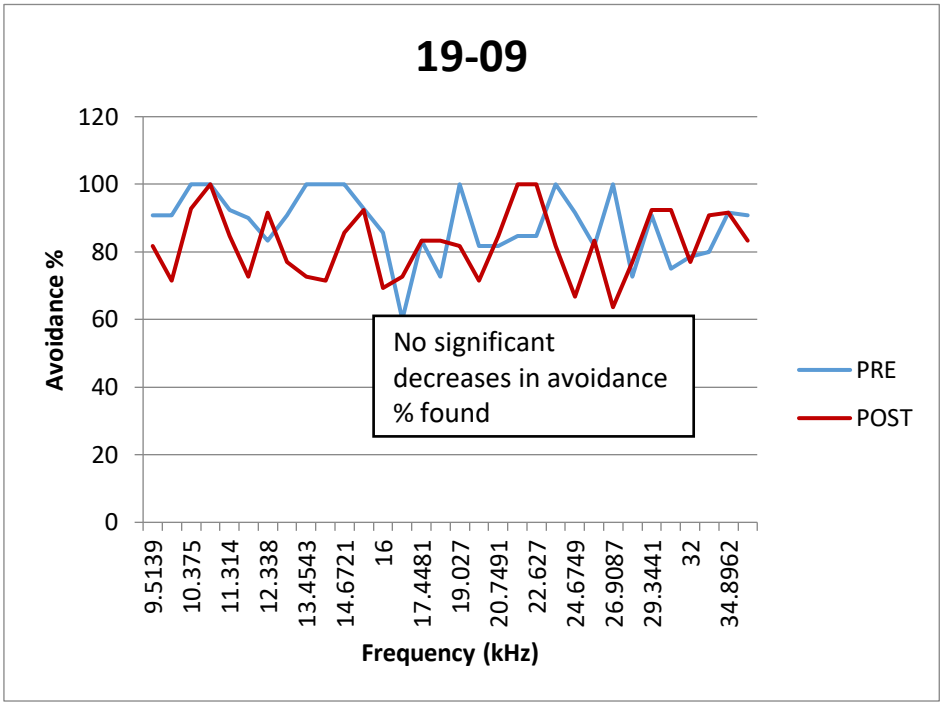


Figure 2.2: *The audiogram and A.A. behavioral results for mouse 19-07. Tinnitus was indicated at 18.33 kHz.*





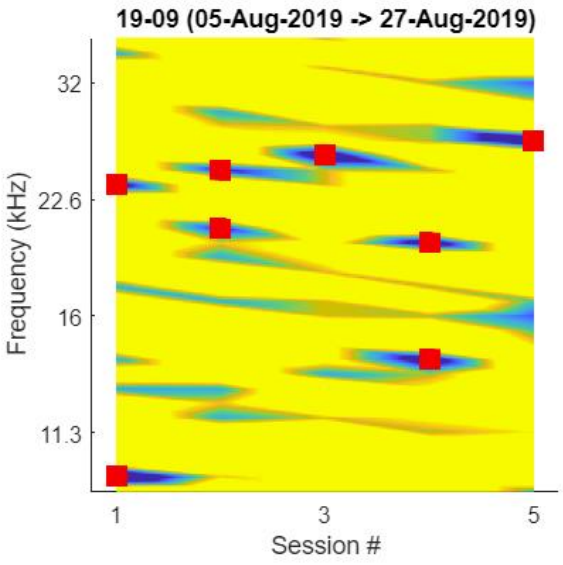
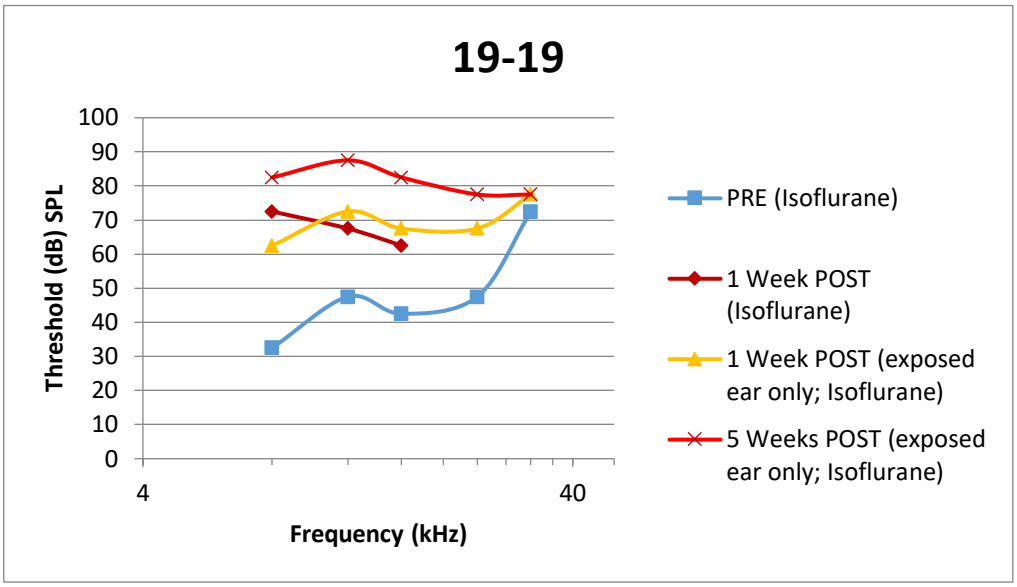
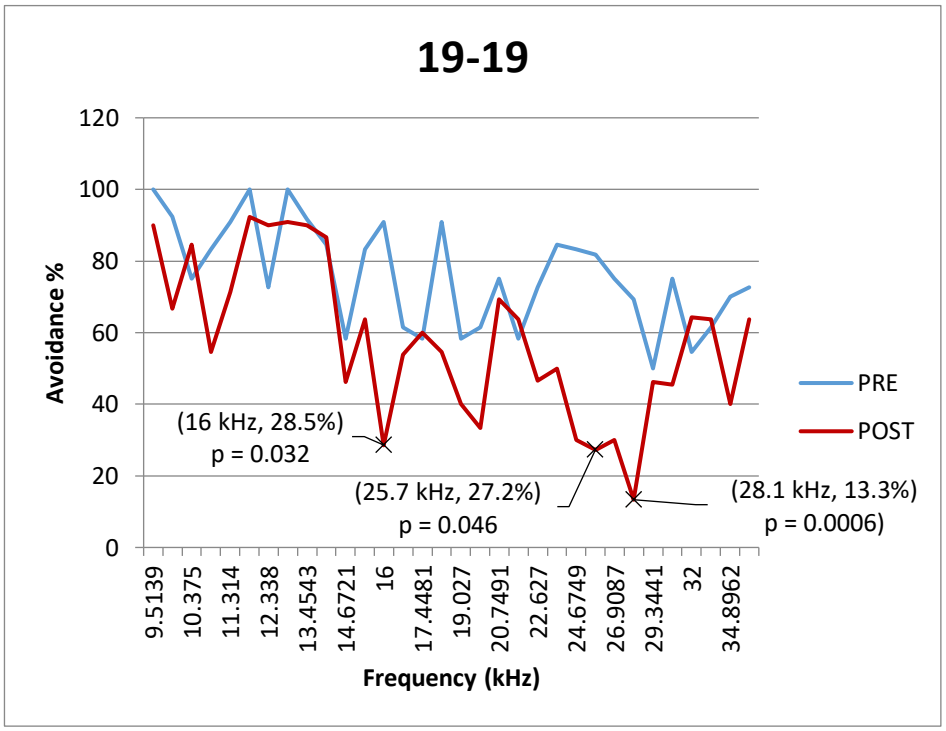


Figure 2.3: *The audiogram and A.A. behavioral results for mouse 19-09. Tinnitus was not indicated by behavioral results.*





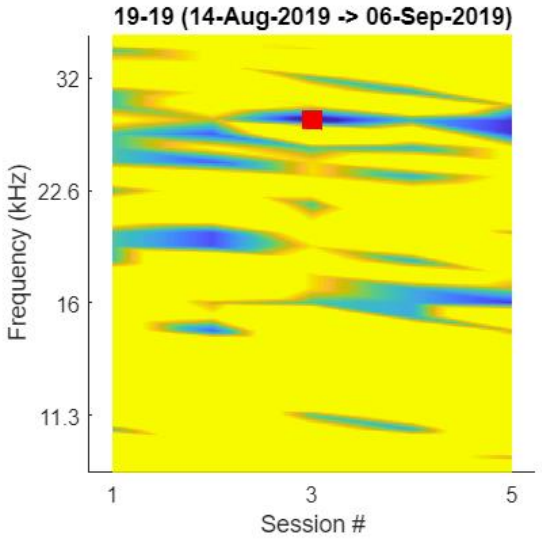
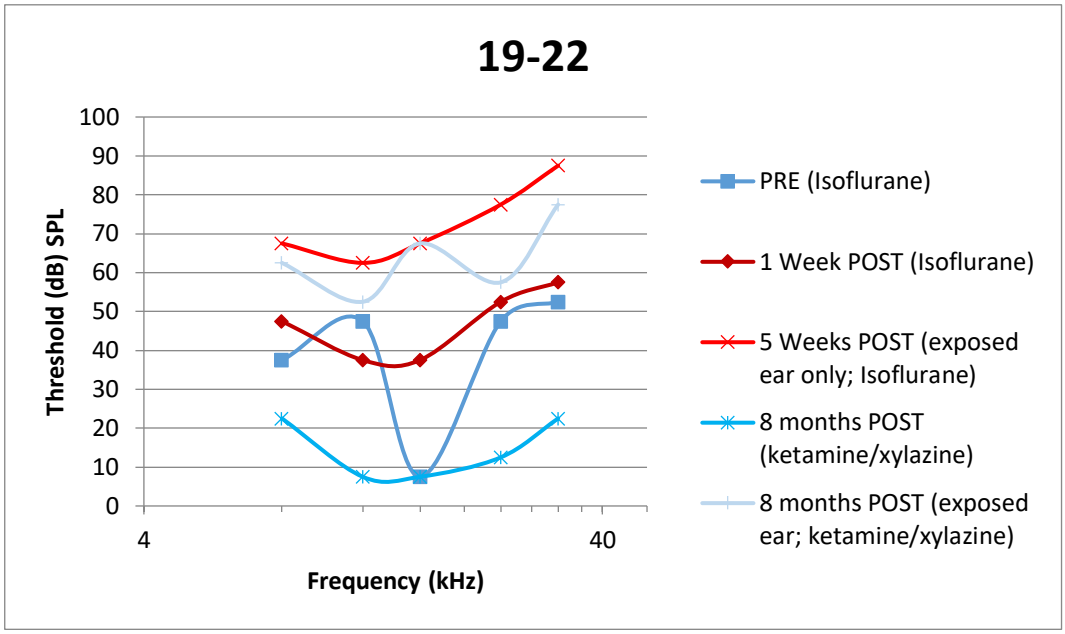
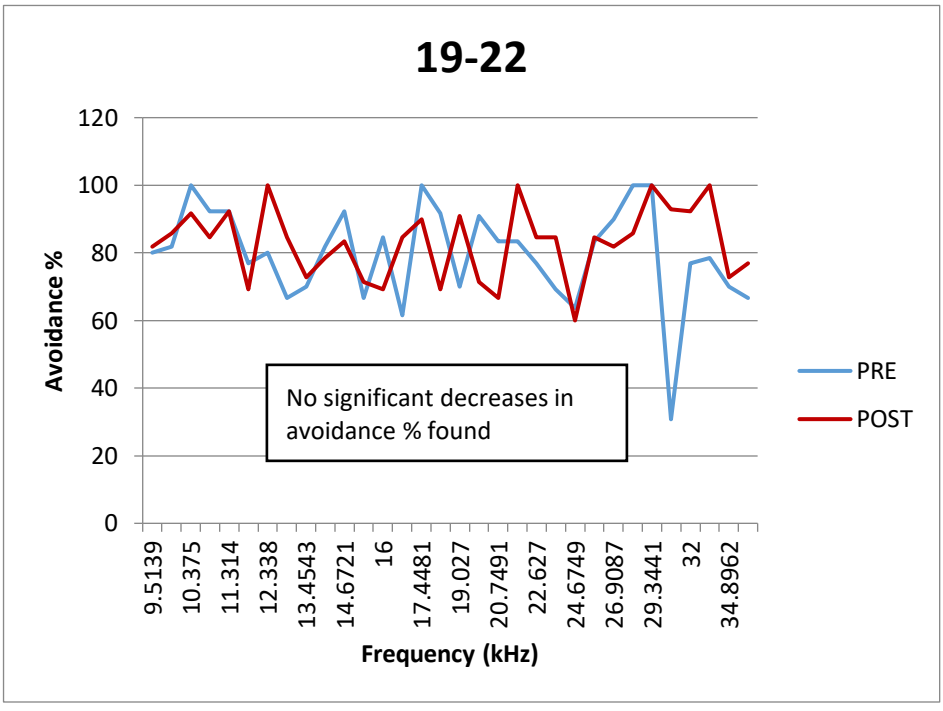


Figure 2.4: *The audiogram and A.A. behavioral results for mouse 19-19. Tinnitus could not be assessed due to elevation in hearing thresholds.*





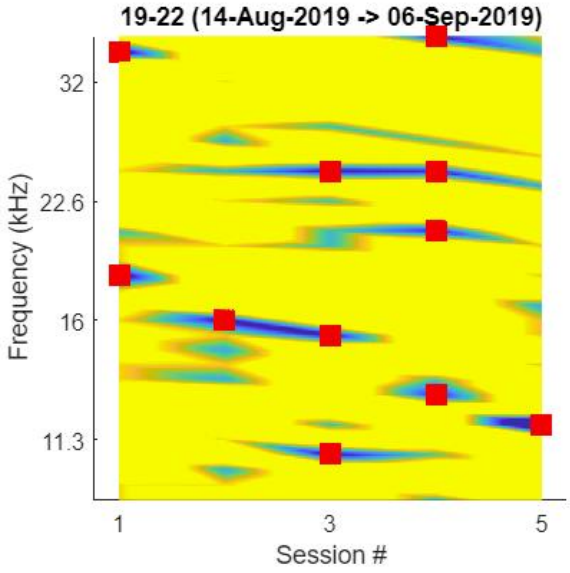
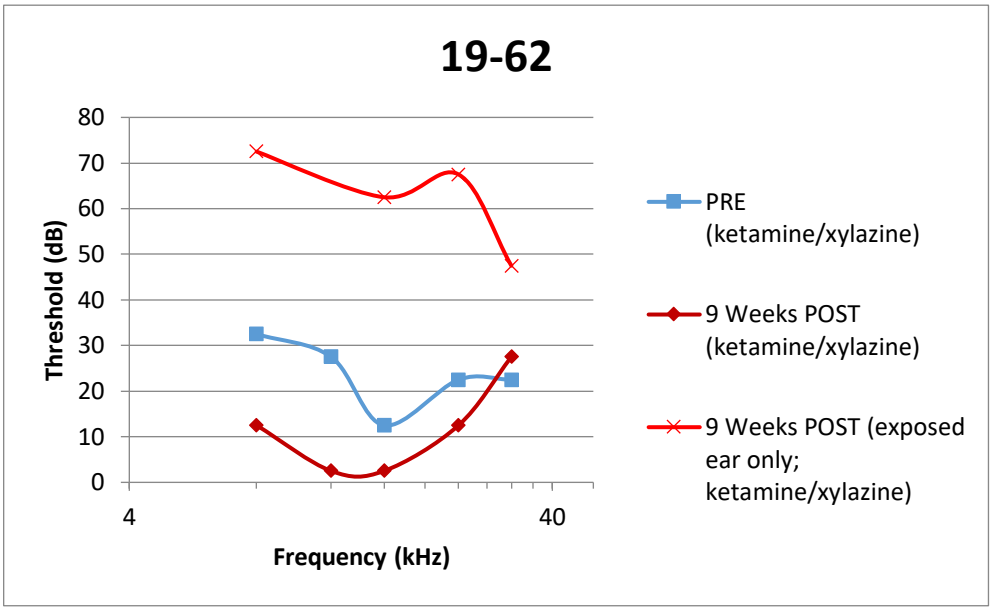
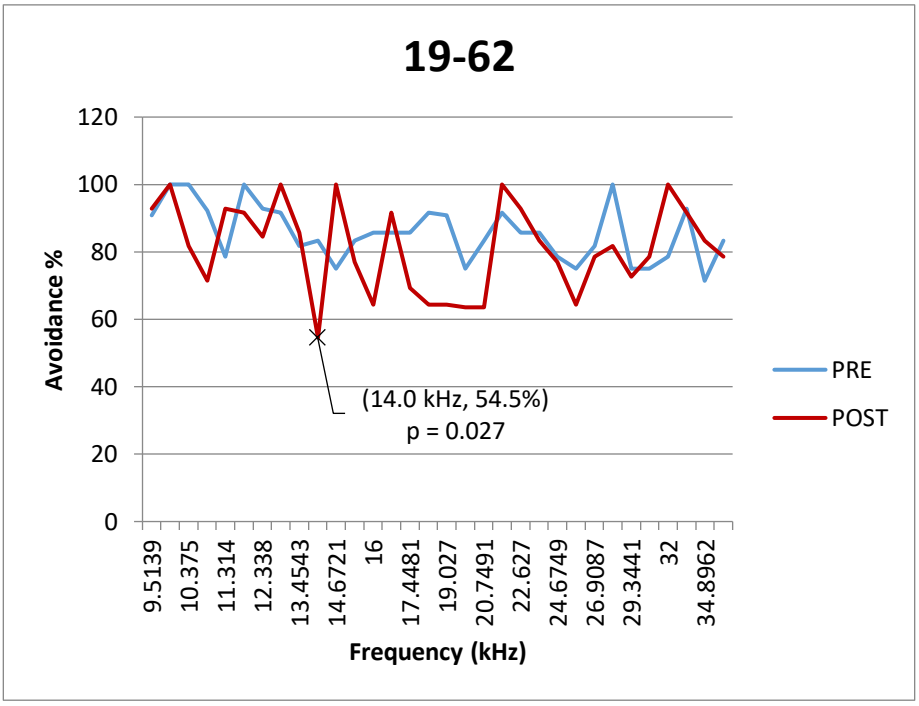


Figure 2.5: *The audiogram and A.A. behavioral results for mouse 19-22. Tinnitus was not indicated by behavioral results.*





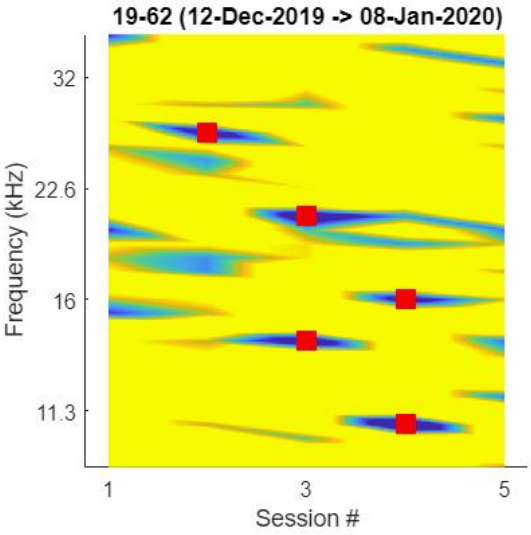


Figure 2.6: *The audiogram and A.A. behavioral results for mouse 19-62. Tinnitus was not indicated by behavioral results.*

Mouse 19-07 was found to have tinnitus, with the frequency of its tinnitus located at 18.33 kHz. Mouse 19-07's ABR thresholds nearly recovered following sound exposure, and its AMFR thresholds were also within normal hearing limits (Figures 2.1 and 2.2). This indicated that 19-07's hearing capabilities were conserved, and deficits in A.A. performance could be attributed to tinnitus, and not hearing loss. A significant decrease in avoidance percentage was observed at 18.33 kHz ($p= 0.0022$), and deficits at this same frequency were present in 3 out of the 5 A.A. sessions (Figure 2.2). Both parameters to classify tinnitus were met, meaning that 19-07 shows behavioral signs of tinnitus at 18.33 kHz.

Mouse 19-09 did not show behavioral signs of tinnitus according to the study's parameters. Although a post-sound exposure ABR threshold was not recovered, AMFR testing indicated that 19-09's hearing thresholds returned to pre-sound exposure levels only 1 week following sound-exposure (Figure 2.1 and 2.2). This indicated that this mouse had conserved hearing capabilities. However, no frequency was found to have a significantly decreased avoidance percentage from the overall POST mean avoidance (Figure 2.3).

Mouse 19-19 could not be assessed for tinnitus. It was evident from a notable increase in the ABR threshold that overall hearing thresholds were elevated (Figure 2.1). AMFR testing also indicated elevated thresholds, with the thresholds of several higher frequencies unable to be tested (Figure 2.4). This elevation of hearing thresholds, especially at high frequencies, was even observed before sound exposure in the AMFR testing. Because behavioral deficits due to tinnitus and hearing loss cannot be differentiated in the A.A. test, 19-19 could not be evaluated for signs of tinnitus.

Mouse 19-22 did not show signs of tinnitus based upon the study's parameters. The mouse's ABR thresholds nearly returned to baseline, and AMFR thresholds were within normal limits 1 week and 8 months following sound exposure (Figures 2.1 and 2.5). Normal hearing capabilities following sound exposure indicated that A.A. performance could be analyzed for signs of tinnitus. However, no significant decreases in avoidance percentage from the overall POST mean could be found. 19-22 did not show behavioral signs of tinnitus.

Lastly, mouse 19-62 also did not show signs of tinnitus. Hearing thresholds following sound exposure were conserved, as was indicated by the ABR and AMFR thresholds (Figures 2.1 and 2.6). Only one frequency, 14.0 kHz, was found to have an avoidance percentage significantly below the overall POST mean avoidance ($p = 0.027$) (Figure 2.6). However, deficits at this frequency was not observed across multiple A.A. sessions.

Based on the study's two parameters, only one out of the five mice exposed to the 116 dB SPL noise centered at 16 kHz with a ½-octave bandwidth was found to have tinnitus. The frequency of tinnitus for this mouse (19-07) was 18.33 kHz.

Mice That May Have Tinnitus, but Did Not Meet Parameters

The two-fold paradigm used in this analysis involved (1) identifying a frequency in the POST A.A. with a mean avoidance significantly below ($p < 0.05$) the POST mean avoidance of all frequencies, and (2) a significantly decreased avoidance percentage ($p < 0.05$) at this frequency in at least 2 out of the 5 A.A. sessions. This paradigm was adopted

to ensure that deficits in the A.A. representing tinnitus were not only found to be different from overall performance, but consistent across multiple days.

While only 2 out of the 9 mice were identified to have tinnitus with this paradigm, it is possible that there were several additional mice that had tinnitus. If a mouse had tinnitus not just at one specific frequency, but a broader range of frequencies, then it is possible that behavioral deficits would appear at a different frequency with each session. For example, if a mouse had tinnitus that ranged from 16-17 kHz, its Surface Profile could indicate significant decreases in performance at 16 kHz for one session, 16.7 kHz for another session, and so on.

The A.A. test presents warning tones at 32 different frequencies. It is possible that if low avoidance percentages were occurring at close, but different frequencies at each session, that the behavioral deficits could have been averaged out among the POST curve. This would result in potential frequencies of tinnitus not being found to be statistically significant with the t-test that was employed. The presence of tinnitus across a range of frequencies has been observed, as well as slight shifting in tinnitus frequencies over time (Longenecker et al., 2014).

With these circumstances considered, it is possible that mice 19-06, 19-09, and 19-54 may have had tinnitus. This possible trend can be seen in Surface Profiles of these mice (Figures 1.2, 2.3, and 1.5).

In its Surface Profile, mouse 19-06 was observed to have a significantly decreased avoidance percentage at 15.3 kHz, which rose to 16 kHz, and then 16.7 kHz over subsequent sessions (Figure 1.2). Although the avoidance percentage at 16 kHz was found to be significantly below the overall mean POST avoidance ($p = 0.0038$),

consistent deficits at 16 kHz were not seen in the Surface Profile because the frequency of the low performance was slightly shifting (Figure 1.2).

Mouse 19-09 was observed to have significantly decreased avoidance percentages at 23.6 kHz, 24.6 kHz, 25.7 kHz, and then 26.9 kHz, all during different A.A. sessions (Figure 2.3). Because these significant deficits in behavioral performance were spread out across different frequencies, their low values were averaged out among the POST curve, and no frequencies were found to be significantly below the overall POST mean avoidance (Figure 2.3).

Mouse 19-54 demonstrated significant decreases in its avoidance percentage at 19 kHz and 18.2 kHz, as well as at 16.7 kHz and 16.0 kHz, all during different A.A. sessions (Figure 1.5). Only 16 kHz was found to have a mean avoidance be significantly below the overall POST mean avoidance with the t-test (Figure 1.5). However, because no two A.A. sessions were found to have a significant decrease in avoidance at exactly 16 kHz, the animal was ruled out for tinnitus.

While the frequency-specific nature of the A.A. test may allow for tinnitus frequencies to be more accurately located, it also presents challenges of what behavioral results can define tinnitus. Such occurrences should be considered in future analyses of A.A. data for tinnitus.

Comparison of Successful Tinnitus Induction Between Sound Exposure Groups

Of the 9 mice sound-exposed and behaviorally tested, only 2 mice met the parameters for showing behavioral signs of tinnitus. The mice observed to have tinnitus

were 19-18 and 19-07. Mouse 19-18 was exposed to the 116 dB SPL noise centered at 16 kHz with a bandwidth of 2 kHz. Mouse 19-07 was exposed to a noise with the same intensity and center frequency, but with a bandwidth of a ½ octave. Mouse 19-18's tinnitus was indicated at 16.7 kHz, while 19-07's tinnitus was observed to be at 18.33 kHz.

Because tinnitus was induced in one mouse from each sound exposure group, it is not possible to determine whether one sound exposure is more effective than the other in inducing tinnitus.

Mice 19-06, 19-09, and 19-54 did not meet the parameters for being classified as having tinnitus, however, as previously discussed, it is possible that these mice may still have tinnitus. 19-06 and 19-54 were exposed to the noise with a 2 kHz-wide bandwidth, while 19-09 was exposed to the noise with a ½ octave bandwidth. If these mice were included in the final cohort of mice with tinnitus, then 3 mice would have tinnitus induced by exposure to the noise with a 2 kHz-wide bandwidth, and 2 mice would have had tinnitus induced by exposure to the noise with a ½ octave bandwidth. Again, these results indicate that neither sound exposure may be more likely to induce tinnitus.

Bandwidth of Sound Exposure and Frequencies of Resulting Tinnitus

Of the mice that were exposed to the sound with a 2 kHz-wide bandwidth, only one met the two-fold paradigm for being classified as having tinnitus. This was mouse 19-18, which demonstrated tinnitus at a frequency of 16.7 kHz (Figure 3.1). Two additional mice exposed to this same sound showed behavioral signs of tinnitus, but did not meet the two parameters described to identify tinnitus. These were mice 19-06 and

19-54. Mice 19-06 showed signs of tinnitus in a frequency range of 15.3-16.7 kHz. Mouse 19-54 showed signs of tinnitus in two frequency ranges: at 16-16.7 kHz and 18.2-19.0 kHz (Figure 3.1).

Of the mice that were exposed to the sound with a ½ octave-wide bandwidth, only one mouse met the two parameters for being classified as having tinnitus. This was mouse 19-07, which was observed to have tinnitus at a frequency of 18.33 kHz. One additional mouse exposed to the sound with a ½ octave bandwidth showed behavioral signs of tinnitus, but did not meet the two parameters discussed. This was mouse 19-09, which showed behavioral signs of tinnitus at frequencies ranging from 23.6-26.9 kHz (Figure 3.1).

A 2 kHz-wide bandwidth centered at 16 kHz ranges from 15-17 kHz, while a ½-octave wide bandwidth centered at 16 kHz ranges from 13.454-19.207 kHz. Mice that were exposed to the sound with a 2 kHz bandwidth appeared to have tinnitus at frequencies closer to the 16 kHz center of the noise than mice in the ½-octave bandwidth group (Figure 3.1)

Sound Exposure Group	*116 dB SPL noise centered at 16 kHz with a 2 kHz-wide bandwidth*			*116 dB SPL noise centered at 16 kHz with a ½ octave wide bandwidth*	
Mouse	19-18	19-06*	19-54*	19-07	19-09*
Frequency of Tinnitus (kHz)	16.7	15.3-16.7	16-16.7 and 18.2-19.0	18.33	23.6-26.9

Figure 3.1: *Comparison of frequencies of tinnitus between the two sound exposure groups. Mice with a * were not found to have tinnitus by the parameters described in the study, but may instead have tinnitus across a broader range of frequencies.*

Overall, it appears that mice exposed to a 1 hour-long, 116 dB SPL 16 kHz sound with a 2 kHz-wide bandwidth may demonstrate tinnitus at frequencies closer to the 16 kHz center. Mice exposed to the same intensity and duration of sound, but with a ½-octave wide bandwidth, seem to show behavioral signs of tinnitus across a broader range of frequencies. More experimentation with larger study groups is needed to further understand these phenomena.

DISCUSSION:

Hearing Threshold Tests

It is important to note that the two anesthetics used for hearing threshold tests both impact the thresholds of mice in different ways. It has been generally observed that isoflurane can elevate hearing thresholds by 20-30 dB SPL, while ketamine/xylazine offers a more accurate representation of thresholds (Ruebhausen, et al., 2012, Kim, et al., 2012). The laboratory group initially used isoflurane for threshold tests due to the convenience and efficiency of the anesthetic. Towards the end of experimentation with this mouse cohort, ketamine/xylazine was used as an anesthetic in order to obtain more accurate thresholds.

Because of isoflurane's consistent elevation of hearing thresholds, the true thresholds of mice were presumed to be 20-30 dB SPL lower than the reported thresholds. Several mice that were initially tested with isoflurane underwent a second AMFR test with ketamine/xylazine following their sound exposures. These additional threshold tests provided more accurate representations of the hearing capabilities of the mice.

Active Avoidance Compared to other Behavioral Tests for Tinnitus

Behavioral tests are the primary method of assessing the presence of tinnitus in animals. However, because this type of Learned Active Avoidance (A.A.) task is relatively new, no studies currently exist that use this paradigm to evaluate tinnitus. Because there is no established method to interpret A.A. results, the two-fold paradigm to identify tinnitus was based off of previous behavioral models and the experimenters' discretion.

The A.A. behavioral test is not the first operant-based conditioning task that has been used to evaluate tinnitus. The first behavioral test involving operant conditioning arose early in the field and involved training rats to lick from a water spout only during periods when sound was playing. If licks continued during the silent periods, the rats would receive a small electric shock. Rats were conditioned to suppress their licking during gaps of silence, and rats with presumed tinnitus were found to struggle with detecting the silent gaps (Jastreboff et al., 1988). This paradigm was later adapted with the opposite parameters: silent periods were "safe" times to drink for rats, and drinking during sound presentation was punished by mild shocks (Jones & May, 2017). Similar models were adapted from this paradigm that involved rats pressing a lever for food pellets. Food was delivered while sounds were playing, but if the lever was pressed during a silent period, a mild shock was delivered (Bauer et al., 2001).

Although behavioral signs of tinnitus have been identified with these previous methods, it is important to note that both of these models involved water and food deprivation. This can cause additional stress for an animal, which can lead changes in

behavior that could confound behavioral test results (Faraco et al., 2014, Brozoski & Bower, 2016).

Currently, the most commonly used behavioral tests for tinnitus does not involve operant-based conditioning, but utilizes an animal's reflexive response to intense sounds. The test is called the gap prepulse inhibition of the acoustic startle reflex (GPIAS) (Galazyuk, et al., 2015, Longenecker, et al., 2011, 2014, 2018). GPIAS differs from A.A. in that it involves no conditioning. Instead, intense sound stimuli are presented at random to a mouse and the magnitude of a mouse's startle response is measured. A continuous background noise is played throughout GPIAS testing. When gaps of silence are inserted right before the startling sound, a mouse braces for the loud stimulus to come. Therefore, the insertion of gaps of silence before a loud stimulus reduces a mouse's startle response. The theory is that mice with tinnitus will have more trouble recognizing these silent gaps because their tinnitus will "fill in" the gap. Therefore, mice with tinnitus are expected to demonstrate different startle response patterns than mice without tinnitus.

GPIAS has been used as a behavioral test for tinnitus in studies using sound-exposure methods similar to this study. After unilateral exposure to a 16 kHz 116 dB SPL noise, lasting from 45 minutes to 1 hour, tinnitus was identified in mice using GPIAS (Longenecker et al., 2011, Sturm et al., 2017, Turner et al., 2012). One study found tinnitus to be induced in 86% of sound-exposed mice, with the tinnitus frequencies predominately presenting at a range of 20-31 kHz (Longenecker et al., 2011). Another study found that approximately 50% of sound-exposed mice showed signs of tinnitus at a range of 16-32 kHz (Sturm et al., 2017). An additional study found behavioral evidence of tinnitus in all 14 of its sound-exposed mice around 10 kHz (Turner et al., 2012).

Although these mice were anesthetized during their sound exposures, these prior experiments support that a 1-hour duration, 116 dB SPL noise centered at 16 kHz is successful in inducing tinnitus.

In this study, potentially 5 out of the 9 sound-exposed mice showed behavioral signs of tinnitus. This indicated >50% tinnitus induction rate, which seems to be consistent with past experimental success in tinnitus induction.

While GPIAS has been widely employed as an animal test for tinnitus, there are still questions regarding its efficacy. Both rat and human studies have indicated that tinnitus may not impact an individual's ability to detect gaps of silence (Boyen et al., 2015, Radziwon et al., 2015). Furthermore, it has been observed that the startle responses of mice and rats may diminish over time in GPIAS. When rodents are repeatedly exposed to a loud stimulus, their startle responses can decrease in magnitude (Lobarinas et al., 2013, Longenecker et al., 2011, Chen et al., 2013). This habituation in GPIAS can be somewhat avoided through either reducing the number of trials a mouse undergoes, or by excluding mice that do not show decent startle responses. However, these practices can decrease that validity and strength of the GPIAS results, as well as possibly eliminate animals that may have tinnitus (Hayes et al., 2014).

The A.A. behavioral test offers a new method of evaluating tinnitus in mice without the added variables of gap-detection, or habituation to loud, startling sounds. A.A. also involves the presentation of 32 different frequencies, allowing for a more precise location of where tinnitus may lie. Additional studies and methods of analyses are still needed in order to assess A.A.'s validity and efficacy and identifying tinnitus.

However, this study presents primary and promising results in how tinnitus could be identified in mice.

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