Detecting Tinnitus: GPIAS Relationships with Hearing in Mice

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Tinnitus is the perception of sounds, including ringing, chirping, among others, in the absence of a physical source inside or outside the body. Curing tinnitus requires a reliable animal model of tinnitus to conduct research. After inducing tinnitus, post-screening to confirm an animal has developed tinnitus is vital – animals cannot be asked. **Gap-prepulse inhibition of the acoustic startle reflex (GPIAS)** is the screening method investigated here. The **acoustic startle reflex (ASR)** is a protective response to sudden, intense noise stimulus, and causes a measurable flinch. In GPIAS, a silent gap is introduced within a constant background tone preceding a noise stimulus. Theoretically, tinnitus "covers" this gap and weakens suppression, indicating the development of tinnitus. However, GPIAS is controversial because whether weaker suppression is caused by tinnitus or hearing loss is unclear. This investigation emulated the covering effect with no-gap startle responses and aimed to clarify this confusion. Three features of ASR were examined in C57BL mice: **ASR peak-to-peak amplitude (ASR_{P-P}), ASR latency (ASR_{LAT}), and ASR duration (ASR_{DUR})**. Percentage change in these features between no-gap and gap startles were calculated, showing if suppression occurred. GPIAS background frequency modulates ASR inhibition. Mapping percentage change in ASR_{P-P}, ASR_{LAT}, and ASR_{DUR} as a function of background frequency, the results suggest a feasible method for modelling C57BL mice hearing sensitivity – a novel approach towards clarifying whether an animal has tinnitus.

INTRODUCTION

Tinnitus is the phantom perception of sound, without a physical source inside or outside the body, that can include ringing, chirping, roaring, among others (Eggermont, 2012). As a prevalent condition (~ 15%) in the population, it is an instigator of depression, anxiety, and suicide. Financially, it causes individuals up to $30 000 in personal economic loss annually, and costs society ~ $26 billion dollars every year ("Tinnitus is an Unacknowledged, Underfunded Public Health Crisis," 2015). There is presently an absence of an effective treatment, and clearly, the development of one is urgently needed. There is currently growing animal research into tinnitus mechanisms, but a major existing problem is accurately determining whether the animals used in research truly have developed tinnitus after the attempted tinnitus induction. This is problematic as animals cannot be simply asked, and proposed solutions to this problem remain controversial.

Various species of animals, including humans, exhibit primitive protective reactions to unexpected noise stimulus – this reflex is known as the acoustic startle reflex (ASR) (Basavaraj & Yan, 2012). ASR is based on neural circuits in the lower brainstem, and when stimulated, is characterized by a flinching/freezing-like constriction of the skeletal muscles (Basavaraj & Yan, 2012). The reflex can be modulated by a stimulus preceding the noise stimulus (Galazyuk & Hébert, 2015). For example, by introducing a silent gap that precedes a noise burst within a constant background tone, the ASR can be attenuated. This is known as gap-prepulse inhibition of the acoustic startle reflex (GPIAS). It is based on how well an animal can detect this gap that GPIAS has been extensively studied as a method to detect the presence of tinnitus in animals. Essentially, in the presence of tinnitus, the embedded silent gap is thought to be filled in, reducing the observed ASR suppression. However, this usage of GPIAS still faces controversy, as whether the observed attenuation of the ASR is truly due to tinnitus is unclear. Hearing loss, a possible consequence of methods meant to induce tinnitus, such as prolonged loud noise exposure, affects GPIAS in a similar way that tinnitus does (Baguley & Fagelson, 2016). That is, if the animal's perception of the background frequency is poor, the embedded silent gap also becomes less perceptible, and thus less effective at inhibiting the ASR (Baguley & Fagelson, 2016). Therefore, the problem with GPIAS is that whether ASR inhibition is due to tinnitus or hearing loss is unclear.

GPIAS can be conducted with a background pure tone. Previous investigations of GPIAS have found that ASR inhibition depends on the frequency of the background pure tone (Steube et al., 2016). The present investigation first aims to map the relationship between background frequency and ASR inhibition. Then, the investigation intends to elucidate any correlations between trends in frequency dependence and previously established trends in hearing sensitivity in the mice used. It is hypothesized that the frequency dependence of ASR inhibition is due to the varying hearing sensitivities of animal subjects at different background

frequencies. At insensitive frequencies, the ability to detect a gap may be limited, causing weaker ASR inhibition. At sensitive frequencies, gap detection should be stronger, causing stronger ASR inhibition. Therefore, a similarity between trends in frequency dependence of ASR inhibition and trends in the animal's hearing sensitivity is predicted. The auditory brainstem response (ABR) threshold measures the minimum intensity of a tone at different frequencies needed for the nervous system to generate an electrical signal in response. It can be used to estimate hearing sensitivity.

The relationship between background frequency and ASR inhibition will be examined by assessing three features of ASR: ASR peak-to-peak amplitude (ASR_{P-P}), ASR latency (ASR_{LAT}), and ASR duration (ASR_{DUR}) (Figure 1). ASR inhibition is found by comparing ASR features in GPIAS startle responses to ASR features in no-gap startle responses, as depicted in Figure 2. No-gap startles were used to emulate the covering effect of tinnitus, as it was deemed that inducing tinnitus was unnecessary procedurally and would also unnecessarily stress the animals.
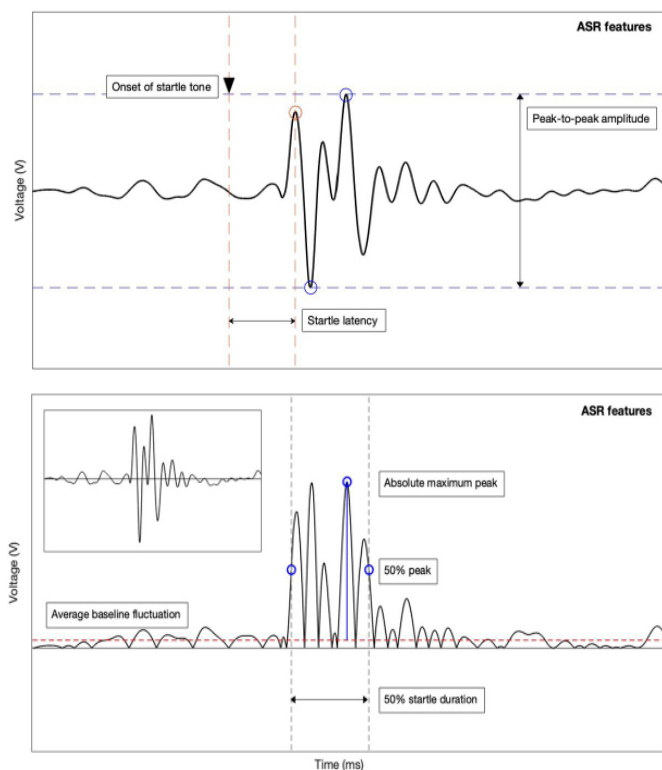

Figure 1. The three ASR features examined depicted in a typical ASR waveform: startle latency (ASR_{LAT}), peak-to-peak amplitude (ASR_{P-P}), and 50% startle duration. Startle latency and peak-to-peak amplitude are depicted in the top illustration. Startle latency (ASR_{LAT}) is the time between the onset of a startle tone and the first peak, measuring how fast a startle response occurs. A short latency reflects a strong startle. Peak-

to-peak amplitude (ASR_{P-P}) is the distance between the largest positive and negative peak, measuring the magnitude of the startle response. A large amplitude reflects a strong startle. Peaks were defined using a MATLAB algorithm. 50% startle duration is depicted in the bottom illustration. Here, the waveform is the absolute value of the ASR waveform from the top illustration.
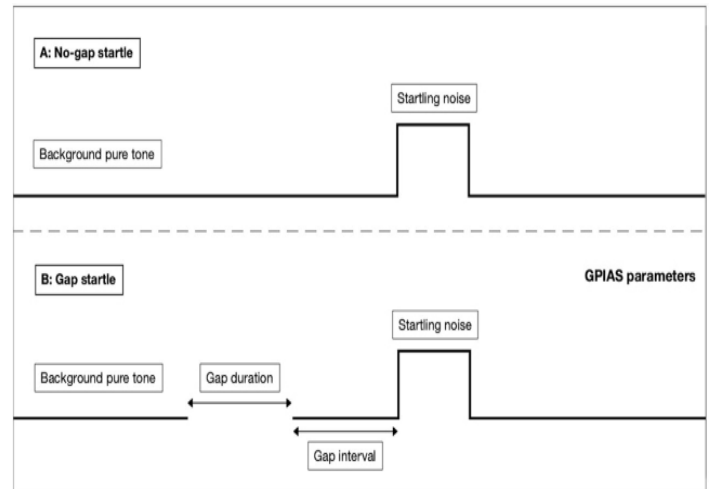

Figure 2. No-gap startle compared to gap startle (GPIAS). In tinnitus, the gap in GPIAS is thought to be covered, in a way similar to the no-gap startle, creating an unsuppressed startle response. Contrarily, GPIAS in the presence of no tinnitus, depicted by the gap startle, inhibits the startle response. The four labelled parameters are pertinent to this investigation and can be manipulated to modulate a startle response.

MATERIALS & METHODS
Materials
Animals
Five female C57BL mice, 4 – 5 weeks in age and weighing between 15 and 21 g were used in this investigation. Experimental procedures were fully in line with the Canadian Council on Animal Care and the Animal Care Committee, University of Calgary. Mouse experiments were all conducted only after receiving IAUTP mouse handling certification and under the supervision of a certified senior member in the Yan Lab for Hearing Research at the University of Calgary.

Equipment
Startle tests were completed with mice placed in a small custom-made animal housing within a dark soundproof chamber. The housing was equipped with four piezoelectric transducers which converted animal movement to voltage signals. Output signals were sampled at 25 kHz and recorded using BrainWare software (TDT). A loudspeaker (87 dB SPL), was placed 10 cm away from the housing for delivering noise bursts that covered a broad range of frequencies and the background tone (67 dB SPL). Experimental setup, depicted in Figure 3, was identical for every mouse.
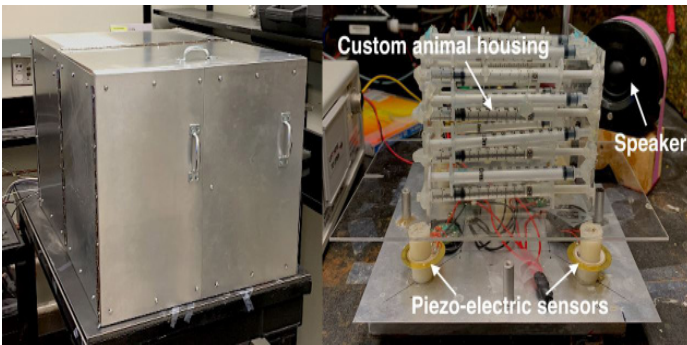
Figure 3. Main apparatus used to conduct startle tests. The left image depicts the animal housing that each mouse was inside for experiments, as well as the speaker and sensors used. The right image is the soundproof chamber containing this apparatus.

Acoustic stimulation for ASR and GPIAS
The experimental procedure consisted of a three-stage process explained below. The objective of this procedure was to compare ASR features between no-gap ASR and GPIAS at varying frequencies to determine how changes in ASR features vary as a function of background frequency.
Stage 1: Setting a baseline - measuring no-gap ASR
Completed individually, each mouse was placed in the housing and was given ~3 minutes to acclimate. Then, the noise bursts were delivered within a continuous background pure tone. Background pure tone frequencies varied logarithmically from 0.5 to 24 kHz covering a broad range, allowing for fewer manipulations which reduced habituation and animal stress. At each frequency, a running average of 60 repetitions of a no-gap startle was conducted to obtain an ASR waveform. Each repetition, or sweep, lasted 1000 ms with the startling noise burst delivered at 300 ms into the repetition. There was a ten-minute break before Stage 2.
Stage 2: Optimizing GPIAS parameters
To obtain the most observable ASR changes in each mouse in Stage 3, a self-devised parameter optimization process was conducted. GPIAS parameters were manipulated individually to determine optimal parameters.
The background frequency producing the strongest ASR_{P-P} found in Stage 1 was used as a control. Controlling this background frequency and controlling gap interval to 50 ms, GPIAS was conducted while manipulating gap duration from 50 to 200 ms, with steps of 25 ms. The gap duration yielding the strongest ASR_{P-P} inhibition was marked as optimal. Controlling gap duration and frequency to their established values, gap interval was manipulated from 25 to 150 ms, with steps of 25 ms. The gap interval yielding the strongest ASR_{P-P} inhibition was marked as optimal. There was a ten-minute break before Stage 3. Between mice, there was clear variance in optimal parameters.

Stage 3: Setting a comparative - measuring GPIAS
Using optimal gap duration and interval, GPIAS was conducted with the same logarithmically distributed background frequencies used in Stage 1. Waveforms were recorded with the same running average procedure, except with a gap introduced prior to the noise burst. Gap duration and interval used varied between mice, due to each mouse having unique optimal parameters.
Data processing
Data was processed in MATLAB using fully automated self-written programs. Here, the three features of the ASR waveforms were extracted for analysis (ASR_{P-P}, ASR_{LAT}, and ASR_{DUR}). Differences in ASR features between no-gap ASR and GPIAS at each background frequency were quantified by calculating percentage change with the following formulas:

The percentage change formula for ASR_{P-P} and ASR_{DUR}: $\left(\frac{ASR_{gap}}{ASR_{no-gap}} - 1 \right) * 100\%$

Percentage change formula for ASR_{LAT}: $\left(1 - \frac{ASR_{gap}}{ASR_{no-gap}} \right) * 100\%$

A negative sign was applied to the percentage change calculation for ASR_{LAT} to ensure that a negative percentage change in each of the three features always reflected inhibition in the ASR.
These percentage change values were graphically analyzed within MATLAB. It was observed that some waveforms exhibited excessive noise, likely caused by other unwanted movements by the mouse during experimentation. This noise needed to be cleaned to obtain accurate ASR_{P-P} measurements, so a Savitsky-Golay filter was applied. This filter was chosen as the algorithm it uses tends to preserve peak locations while eliminating noise – necessary for preserving measurement accuracy when analyzing ASR_{LAT} and ASR_{DUR} (Yang et al., 2009). The peak locations in ASR waveforms to determine ASR_{LAT} were found by using a built in MATLAB peak finding algorithm. The 50% peak locations used to determine ASR_{DUR} were found with a custom peak finding algorithm.
RESULTS
Determining the influence of background frequency
Prior to further analysis of the data, whether the background frequency influenced the magnitude of ASR inhibition needed to be confirmed. The change in ASR features in individual mice suggested inhibition (Figure 4). Graphically, ASR inhibition was evident in the population and varied with different frequencies (Figure 5), except for a few trials. Combined, this confirmed that

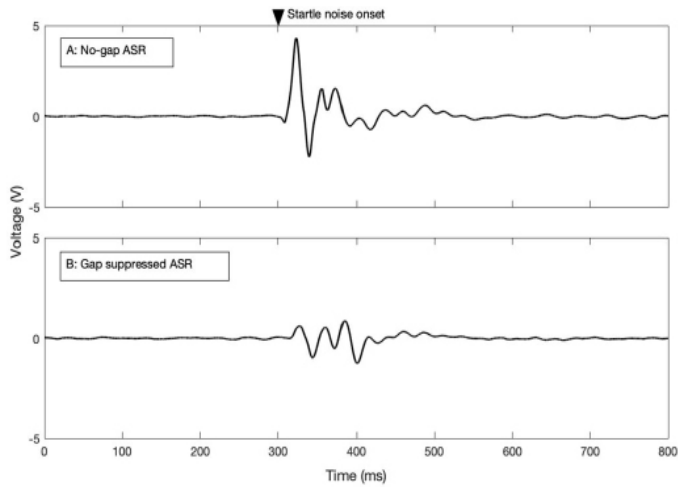background frequency influenced the magnitude of ASR inhibition.

Figure 4. An example of suppressed ASR in mouse 1 due to the presence of a silent gap preceding the startle noise. Background frequency is 4 kHz. The arrowhead represents where the loud startling noise begins.
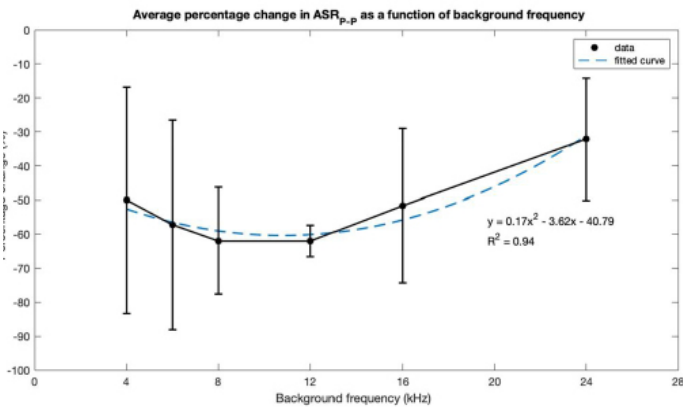

Figure 5. Average percentage change in ASR_{P-P} across all five mice. A parabolic trend resembling ABR thresholds of C57 mice is visible. Error bars represent standard deviation.

Data was then interpreted on a population level. However, because optimized gap parameters were different between mice as mentioned earlier, individual mice were also analyzed to further interpret the data.

Population level
For each feature of ASR, percentage change data from all mice was averaged across each frequency and plotted as va function of frequency ≥ 4 kHz. Pilot mouse data was included since the procedure was the same. Data below 4 kHz consistently showed large variation and was excluded graphically to reveal trends. In ASR_{P-P} and ASR_{LAT} line graphs, a concave parabolic pattern emerged. This pattern resembles trends in ABR thresholds for

C57 mice and the significance of this will be discussed further later (Zhu et al., 2007).
Individual mice
Graphically, for mice 2 and 3, the percentage change in both ASR_{P-P} and ASR_{LAT} reflected the concave parabolic trend (mouse 3 in Figure 6). Fitting their graphs with parabolic lines of best fit revealed high R^2 values ranging from 0.70 to 0.96. This indicated a good fit on the basis of low variance. Because these trends also revealed similarity to C57 ABR thresholds, a question arose: are the ASR_{P-P} and ASR_{LAT} inhibition trends in individual mice consistent when compared to each other? To answer this, trend similarity was estimated mathematically with the derivative.
Estimating trend similarity with the derivative
The derivative describes the sensitivity of a function's output values to changing input values. Deriving the general quadratic formula ($y = ax^2 + bx + c$) produces $y' = 2ax + b$. The coefficient, "2a", determines sensitivity to change – visually, it corresponds to parabola width. Therefore, if the "2a" coefficients in two derived parabolas are similar, their shape/trend will also be similar.

Fitted parabolic trends ($R^2 > 0.70$) for ASR_{P-P} and ASR_{LAT} inhibition in mouse 2 and 3 were derived, and then their coefficients were compared by calculating percentage difference. Mice 2 and 3 yielded coefficient differences of $\sim 40\%$ and $\sim 2\%$ respectively. The very small difference of $\sim 2\%$ between ASR_{P-P} and ASR_{LAT} in mouse 3 is depicted in Figure 6. Percentage difference in parabola vertices was calculated too. Mice 2 and 3 yielded a vertex difference of $\sim 1\%$ and $\sim 40\%$, respectively. ABR thresholds of C57 mice are not perfectly parabolic and neither was the data presented here. However, because the parabolas fit well for
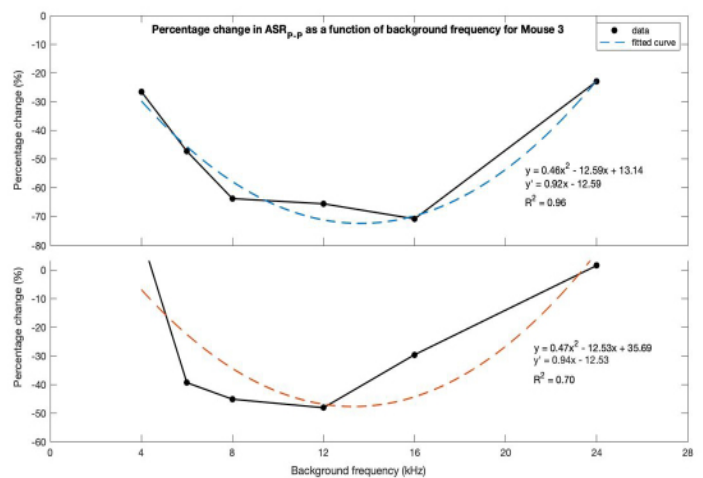

Figure 6. Percentage change trends in ASR_{P-P} and ASR_{LAT} in mouse 3. Highly similar parabolic trend shapes between these two features, both graphically and mathematically from using the derivative, are observed. Again, there is a resemblance to C57 ABR threshold trends.

mouse 2 and 3, this method of calculus was deemed reasonable for estimating trend similarity in these mice.

A weaker parabolic trend appeared in mouse 4 for ASR$_{LAT}$ ($R^2 = 0.44$) and ASR$_{DUR}$ ($R^2 = 0.34$). No significant parabolic trends were observed in mouse 1 and 5.

DISCUSSION

The purpose of this investigation was to assess the effect of background frequency on percentage change in three ASR features, and to understand any similarity between this frequency dependence and the ABR threshold trends in C57 mice. On both the population and individual mouse level, a parabolic trend emerged between background frequency and ASR inhibition. This trend corresponded to ABR thresholds in C57 mice previously measured in other labs. This suggests that C57 mice hearing sensitivity actively affects gap detection and thus corresponds to the frequency dependence of ASR inhibition, as was hypothesized. At insensitive frequencies where ABR threshold is high, it may be difficult to distinguish a gap within a background tone, causing the gap to lose its inhibiting effect. At sensitive frequencies, a gap may be more clearly defined, thus strengthening ASR inhibition. This is a possible reason for why the frequency dependence of ASR inhibition correlated to the patterns observed in C57 ABR thresholds.

Similar trends

Upon the observation that the frequency dependence bore similarities to ABR threshold trends, a question arose of whether the frequency dependence remained consistent in shape and pattern between ASR features. In mouse 3, the trends between ASR$_{P-P}$ and ASR$_{LAT}$ were quite similar, adding support to the idea that the GPIAS trends are modelling hearing sensitivity. That is because under the assumption that hearing sensitivity is causing this frequency dependence, hearing sensitivity should remain the same between features and therefore frequency dependence should remain similar as well. Mouse 2 however, showed larger differences that did not agree with mouse 3 observations, and whether this invalidates the interpretations of the results or was simply due to experimental variation needs to be further explored. The varying vertex height differences between ASR$_{P-P}$ and ASR$_{LAT}$ in mouse 2 and 3 may suggest systematic differences in frequency dependence of ASR inhibition between mice.

Despite the conclusions drawn from these results that observed frequency dependence seems to correlate with the ABR threshold trends of C57 mice, it is important to understand that such patterns did not emerge in all mice or all features of ASR. This variance means that the conceptual insight these results reveal should only be considered as initial results, and further testing is needed.

FUTURE DIRECTIONS & IMPROVEMENTS

The observation in this investigation that frequency dependence of ASR correlates with hearing sensitivity/ABR thresholds seems to have been reported only once in a study that used a different strain of mouse with narrowband noise centred around particular frequencies rather than the pure tones used in this investigation (Longenecker & Galazyuk, 2012). Despite the procedural differences, this encourages continued research into the results and interpretations that this investigation proposes. Replicating this experiment in the future with a larger sample size and more trials is needed to investigate whether the trends in this experiment are true and appear broadly. If those results confirm that ABR thresholds do correlate with the frequency dependence of ASR inhibition, then this relationship may aid GPIAS in determining whether an animal has tinnitus or hearing loss. At that stage, an animal would likely need to fulfill two requirements in order to be considered as having tinnitus:

a) The animal must demonstrate ASR inhibition during GPIAS testing, which is the only metric currently used to determine whether an animal has tinnitus.

b) The frequency dependence of ASR inhibition observed in the animal must correlate with the animal's ABR thresholds.

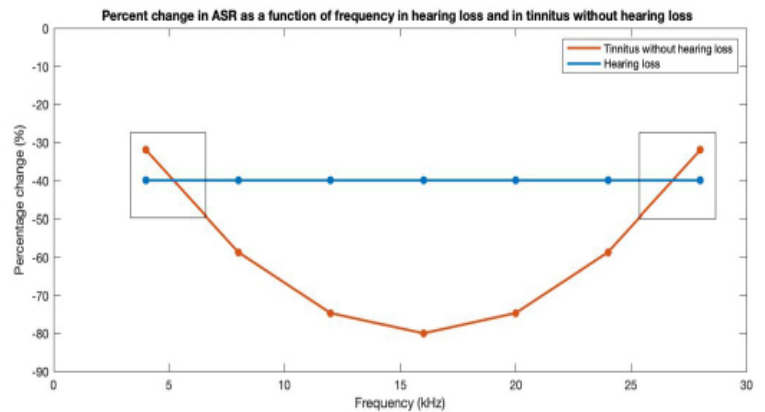This idea is demonstrated in Figure 7 below. While hearing

Figure 7. A hypothetical, generalized comparison of frequency dependence of ASR inhibition in tinnitus vs. hearing loss. The orange trend matches normal ABR threshold trends, the blue trend does not. There can be confusion when determining whether inhibition is due to tinnitus or hearing loss. Here it is shown how both tinnitus and hearing loss can weaken ASR inhibition, and the two rectangular boxes depict how gap inhibition may be similar at particular frequencies, causing further confusion. Under the idea posed by this investigation, that GPIAS trends may model hearing sensitivity, this problem may be alleviated if GPIAS is tested broadly across a range of frequencies. Frequency dependency trends that deviate from an animal's typical hearing sensitivity trends may indicate that inhibition is due to hearing loss, rather than tinnitus. GPIAS trends that follow the normal hearing sensitivity, like the ones found in this investigation, may then reflect tinnitus - thus distinguishing tinnitus from hearing loss.

loss and tinnitus are both causing ASR inhibition, the frequency dependence of ASR inhibition in hearing loss does not correlate with the animal's normal ABR thresholds, suggesting that it does not have tinnitus. Based on an extensive search of the tinnitus literature, the application of this correlation as a method of improving tinnitus detection seems to be novel and may serve as a new direction in detecting tinnitus in animals to assist research developments in the pursuit of a tinnitus cure.

CONCLUSION

Since 2006 when Turner et al. introduced GPIAS as a new behavioural method of detecting tinnitus in animals, it has been widely accepted and used, as it offers simplicity over traditional conditioning procedures (Eggermont, 2012). However, in recent years it has faced scrutiny due to confusion in whether it is detecting tinnitus, or hearing loss. This is a crucial distinction to make for animal research into tinnitus treatments to proceed. The present investigation suggests that a mouse's frequency dependency trends in ASR inhibition may suitably model the mouse's ABR threshold trends, which can therefore indicate if a mouse has tinnitus or hearing loss. Validated well, this proposed novel method of distinguishing tinnitus would be easily implemented in current procedures of GPIAS – one would simply need to repeat GPIAS tests across a broad range of background frequencies to reveal trends in frequency dependence of ASR inhibition.

ACKNOWLEDGEMENTS

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RITCHIE YU

Ritchie Yu is a graduating high school student who will be attending McGill University. After developing tinnitus at a young age, Ritchie experienced the frustration of living with an incurable and under-researched condition - a frustration that many have unfortunately been burdened with for even longer than he has. Given this, he wanted to see what he could do. From conducting global studies to now improving tinnitus diagnosis in the lab, Ritchie aims to positively impact tinnitus research. He hopes that one day, his efforts may help millions around the world hear the sound of silence again.