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# Effect of age on ABRs in mice with EphA4 mutations

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# Effect of Age on ABRs in Mice with EphA4 Mutations

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## Abstract

It is known that EphA4 can influence the establishment of tonotopic pathways in the auditory system. This can be measured by an increase in thresholds on the auditory brainstem response test (ABR) in mice. It is also known that the aging population in humans tends to have poorer thresholds in the high frequency sounds as they age, termed presbycusis or age-related hearing loss. The C57BL/6J background strain of mice that is known to experience a presbycusis-like process, although it is not specified when this process begins and how it progresses through their life span. The goal of this study was to determine how the EphA4 mutation on a C57BL/6J background strain can affect the hearing of mice at different ages. ABR measures were recorded in mice between 2-9 months of age with and without the EphA4 *lacZ* mutation. Analysis of ABR threshold showed similar results to previous studies for mice with the homozygous mutation ($EphA4^{lacZ/lacZ}$). These mice have a rapid decrease in hearing starting early in life; our mice exhibited severe hearing loss even as young as 2 months of age. When we analyzed threshold data for the heterozygous and wild type mice, we found that mice with the heterozygous mutation ($EphA4^{lacZ/+}$) had some preservation of their hearing thresholds using a mid-frequency stimulus (8kHz tone pip) when compared to their wild type littermates ($EphA4^{+/+}$). When using a high-frequency stimulus (12kHz tone pip), both groups had equally poor thresholds. This suggests a heterozygote advantage which preserves their mid-frequency hearing longer and slows down the process of presbycusis. This may have implications for potentially delaying the process of human presbycusis and preserving hearing into later ages.

# Introduction

## Presbycusis

Age related hearing loss, or presbycusis, is a slow decline in hearing thresholds, typically starting at the high frequencies, that is a part of the normal aging process. Many factors, including noise exposure and genetics, can contribute to this process (Gates & Mills, 2005). It is difficult to study this process and the factors that can contribute to it in humans, as presbycusis can take decades to fully develop. Therefore, it is often prudent in this realm of research to use animal models to effectively investigate the process of presbycusis in an abbreviated period of time.

Rodent species have been chosen as models for human presbycusis because of their shorter life spans. This allows researchers to study age related hearing loss over the course of months, rather than years. Mice can be used to understand genetic aspects of hearing loss, as their genomes can be bred to specifically investigate possible genetic causes of hearing loss.

## Age related hearing loss and mice

Mice of the C57BL/6J strain have been used as models for human presbycusis, or age related hearing loss, as they show a similar effect of decreased hearing thresholds, starting in the higher frequencies, at later ages. Certain strains of mice, specifically the C57BL/6J strain, have been used specifically to study this effect. The decline in hearing thresholds is accelerated in mice with this background strain (Henry & McGinn, 1992; Miko, Henkemeyer, & Cramer, 2008; Noben-Trauth & Johnson, 2009). There is not a

definitive age at which the mice can be said to begin to lose their hearing. The inconsistencies in the literature about this time period make it difficult to pin down a specific age or reason for when this process begins. Most studies are conducted when the mice are very young and do not examine the aging process specifically (Henry & McGinn, 1992; Johnson, Erway, Cook, Willott, & Zheng, 1997; Miko et al., 2008; Ohmen et al., 2014).

Although the gold standard for human audiometric evaluations is the behavioral hearing test, the frequency thresholds of mice are typically determined by objective, physiologic means. The auditory brainstem response has been reliably used in mouse research to determine these thresholds (Henry & McGinn, 1992). The range of hearing for a mouse tends to be of a much higher frequency range than is typically tested with human hearing. For example, human hearing spans the frequencies of approximately 20 to 20kHz and mouse hearing spans the frequencies of 1 to about 100kHz (Heffner & Heffner, 2007).

**EphA4 function and auditory research in mice**

The Eph class of proteins is part of the tyrosine kinase receptor proteins which are responsible for several developmental processes (Kullander & Klein, 2002). The function of Eph proteins has been widely investigated through animal studies. EphA4 protein is expressed in the auditory system throughout the inner ear and auditory brainstem. In the mouse, EphA4 is highly expressed in the spiral ligament (van Heumen, Claxton, & Pickles, 2000). Its purpose has been investigated through anatomical and functional

studies (Cramer, 2005; Huffman & Cramer, 2007; Kullander & Klein, 2002; Wallace, Harris, Brubaker, Klotz, & Gabriele, 2016). EphA4 may play a role in keeping cell boundaries consistent and in organizing neural development (Pickles, Claxton, & van Heumen, 2002). Functionally, this may result in higher (poorer) hearing thresholds for the mouse in which EphA4 has been genetically knocked out.

Auditory research using animal models allows the researcher to influence variables that they would otherwise be unable to explore and manipulate. The EphA4 protein is not a mutation that is typically isolated in humans. However, it may be a contributing factor to genetic causes of hearing loss. Therefore, it is important to investigate this protein directly to determine its contribution to the auditory system.

**General Statement of the Problem**

It is known that EphA4 mutations can cause hearing loss in the mouse (Gabriele et al., 2011; Miko et al., 2008; Zheng, Johnson, & Erway, 1999); however, it is unknown exactly when this process begins. In this study, we define a more specific time range in which this process may occur. By testing mice longitudinally over different age ranges, we specify which month presbycusis starts to occur. Although most mouse studies are done when the mice are extremely young and have not started the presbycusis process, it may be important to more narrowly define this range if the genetics in question are involved in the aging process.

Hypotheses:

1) Mice with an EphA4 homozygous mutation will have more hearing loss at earlier ages when compared to heterozygous and wild type animals of the same strain.

2) Mice with the heterozygous EphA4 mutation will have similar thresholds and show a relatively normal amount of age-related hearing loss when compared to their wild type counterparts.

## Methods

Subjects were 45 C57BL/6J mice. The first round of testing included 41 mice (5 were eventually excluded because of inability to complete genotype testing). Ages of these mice ranged from 2 to 9 months. Twenty-six of the mice were wild type, EphA4$^{+/+}$ genotype; 5 mice had a heterozygous mutation, EphA4$^{lacZ/+}$; 5 mice had the homozygous mutation, EphA4$^{lacZ/lacZ}$. The second round of testing included 9 mice that were tested once a month for 3 months (at 6, 7, and 8 months of age). Six of the mice were of the wild type variety and 3 of the mice had the heterozygous mutation.

The combined ages and mutations from both rounds of testing are displayed in the tables below. All procedure followed the current Guide for Animal Care and were reviewed, approved and monitored by the James Madison University Institutional Animal Care and Use Committee (IACUC Protocols #A12-12 and #A16-01).

The first round of testing involved testing each mouse one time. Mice were selected based on variety in age. Mutation type was not known before testing. The second round of testing was done to verify a possible finding after the first group's data was analyzed. These mice were tested longitudinally (once a month for 3 months) at 6, 7, and 8 months of age. Mutation type was also unknown before testing for these mice.

| Mouse Age (months) | EphA4$^{+/+}$ | EphA4$^{lacZ/+}$ | EphA4$^{lacZ/lacZ}$ | **Totals** |
|---|---|---|---|---|
| 2 | 1 | 2 | 3 | **6** |
| 3 | 5 | 3 | 0 | **8** |
| 4 | 10 | 0 | 0 | **10** |
| 5 | 2 | 0 | 1 | **3** |
| 6 | 1 | 0 | 0 | **1** |
| 7 | 0 | 0 | 1 | **1** |
| 8 | 0 | 0 | 0 | **0** |
| 9 | 7 | 0 | 0 | **7** |
| **Totals** | **26** | **5** | **5** | **36** |

Table 1: Mice from first round of testing

| Mouse Age (months) | EphA4$^{+/+}$ | EphA4$^{lacZ/+}$ |
|---|---|---|
| 6 | 6 | 3 |
| 7 | 6 | 3 |
| 8 | 6 | 3 |

Table 2: Mice from second round of testing

| Mouse Age (months) | EphA4$^{+/+}$ | EphA4$^{lacZ/+}$ | EphA4$^{lacZ/lacZ}$ | **Totals** |
|---|---|---|---|---|
| 2 | 1 | 2 | 3 | **6** |
| 3 | 5 | 3 | 0 | **8** |
| 4 | 10 | 0 | 0 | **10** |
| 5 | 2 | 0 | 1 | **3** |
| 6 | 7 | 3 | 0 | **10** |
| 7 | 6 | 3 | 1 | **10** |
| 8 | 6 | 3 | 0 | **9** |
| 9 | 7 | 0 | 0 | **7** |
| **Totals** | **44** | **14** | **5** | **63** |

Table 3: Number of mouse test sessions combined from first and second round

Before auditory brainstem responses (ABR) were recorded, mice were anesthetized with an IP or IM injection of 150 mg/kg ketamine and 30 mg/kg xylazine. They initially received a 2/3 dose based on their weight and received an additional 1/3 dose as needed to maintain anesthesia. Mice were then placed in a sound proof booth on a temperature control blanket. An infrared camera was used to monitor the mice inside the booth while testing. Subcutaneous needle electrodes were placed at the vertex (non-inverting), mastoid (inverting), and back (ground). Impedances were measured and accepted if all locations were $\leq 1k\Omega$.

Electrodes were connected to the Tucker Davis RA4PA 4-channel pre-amp. Stimuli were delivered through a Y-shaped closed tube delivery system and presented to one ear of the mouse. Stimuli were presented using a TDT EC1 high-frequency electrostatic speaker and were monitored using an Etymotic research ER-7C probe

microphone. Stimulus peak frequency, amplitude, and bandwidth were monitored in real time using an Agilent 35670A Dynamic Signal Analyzer to ensure consistency and correct calibration in stimulus presentation.

BioSig software was used to generate three stimuli: an 8kHz tone pip, 12kHz tone pip, and a 100 microsecond broadband click. Presentation of the stimuli was randomized. Threshold was determined by presenting successively lower intensity stimuli which were nominally 90, 70, 60, 55, 50, 45, 40, 35, 30, 25, and 20 dBSPL. Stimuli were presented at a rate of 39.1 clicks/second. Tone pips were 5 msec in duration (0.5 ms rise/fall time and 4 msec plateau) and shaped using a Blackman envelope. Broadband clicks were 0.1 msec in duration. Response was filtered from 300-3000 Hz. Four replication runs were performed (2 condensation and 2 rarefaction) of 100 sweeps each. ABR responses for a one channel recording were recorded using the BioSig software and off-line analyzed.

Off line analysis was performed using a locally written MATLAB program. ABR tracings were analyzed to determine threshold, prominent peak latencies, and peak amplitudes. First, the software displayed two tracings (sum of condensation and rarefaction runs) overlaid on each other. Two independent observers, each naïve to the age and genotype of the mouse, decided if those two tracings showed replicable evidence of an ABR. If yes, the program displayed a similar pair of tracings at the next lower intensity and the operator made a similar decision as to the appearance of any replicable ABR waveform. This continued until the observer decided that there was no response. Threshold was determined as the lowest intensity level at which prominent peaks were able to be visually identified and the two tracings were replicable. Mice with no identifiable threshold at 90 dBSPL were assigned a threshold of 95 dBSPL. The program

then displayed an average of all 4 waveforms through all levels deemed to be above threshold, and the observer clicked on 7 points to represent peaks I through V and the valleys of peaks I and II. If wave V was determined to not be present, the observer clicked on a specific quadrant to indicate the absence of this wave.

Waves I-V were chosen by at least 2 independent observers after training was given on the software and basics of peak picking (including normative latency ranges of the peaks and identifying good versus bad replicability).

Tail snips were taken from each mouse after ABR testing was complete and while the mouse was still anesthetized. Genotyping was done with the tail snips via PCR analysis using the method described in Wallace, et al. (2016). Mice were categorized as homozygotes ($EphA4^{lacZ/lacZ}$), wild types ($EphA4^{+/+}$), or heterozygotes ($EphA4^{lacZ/+}$).

## Results

All ABR tracings were analyzed by at least two independent observers to ensure that the subjective visual detection task was replicable and accurate. The results were analyzed for reliability in threshold, P1 latency, P5 latency, and P1 amplitude choices. There was good correlation for all measurements except P5 latency. Figures 1-4 show correlation coefficients for the following variables: Figure 1: threshold ($r^2$=.98), Figure 2: P1 latency ($r^2$=.82), Figure 3: P1 amplitude ($r^2$=.77), and Figure 4: P5 latency ($r^2$=.17).

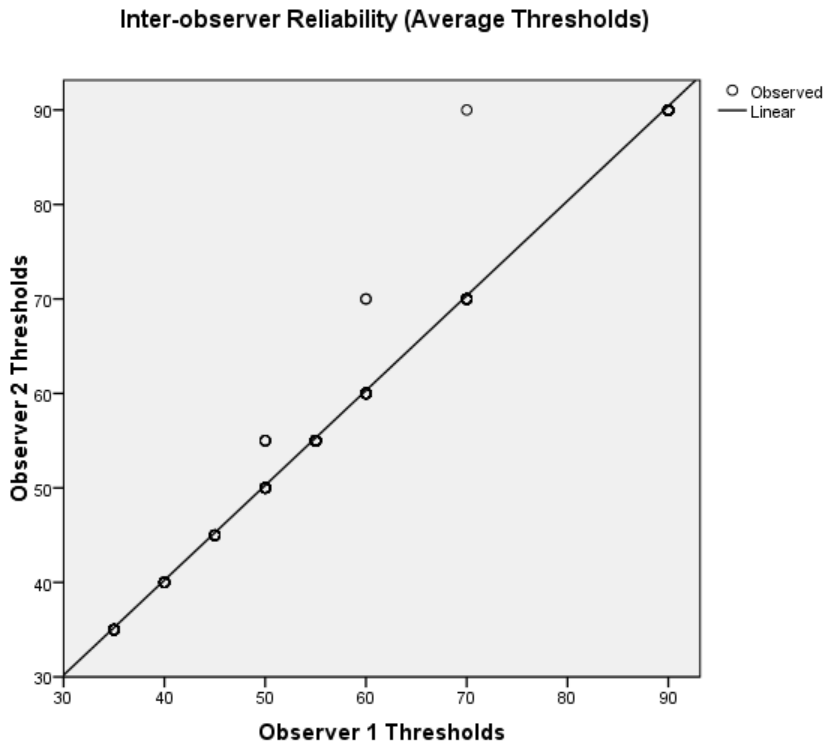


Figure 1: Scatterplot showing relationship between observer 1's threshold choices and observer 2's threshold choices ($r^2$=.98).

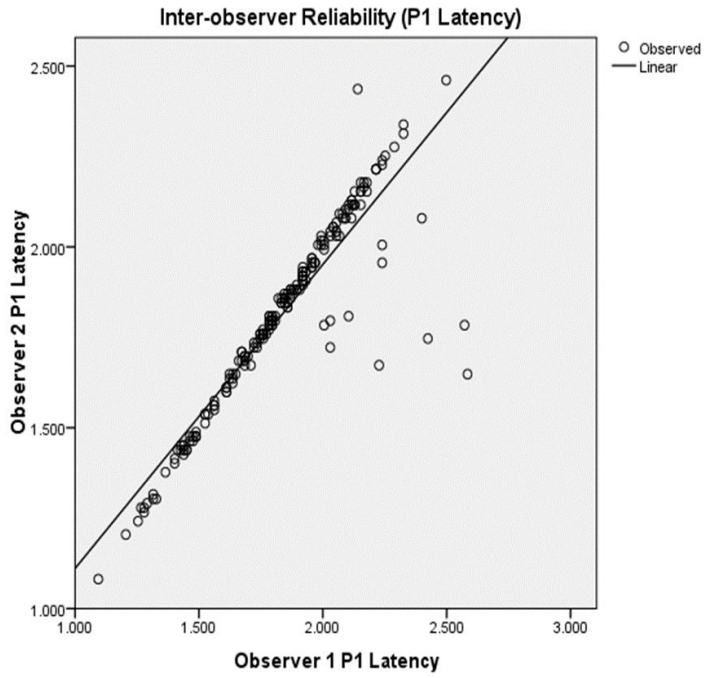


Figure 2: Scatterplot showing relationship between observer 1 and observer 2 in choosing P1 latency ($r^2$=.82).

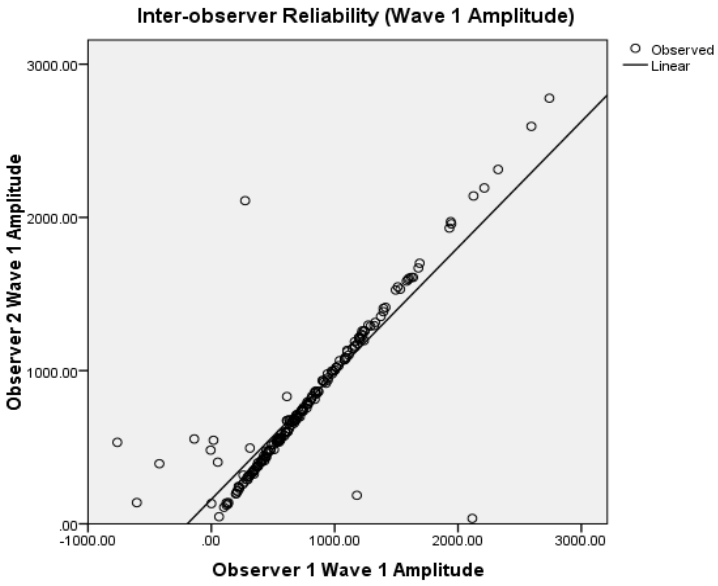


Figure 3: Scatterplot showing relationship between observer 1 and observer 2 in calculating wave 1 amplitude ($r^2$=.77).

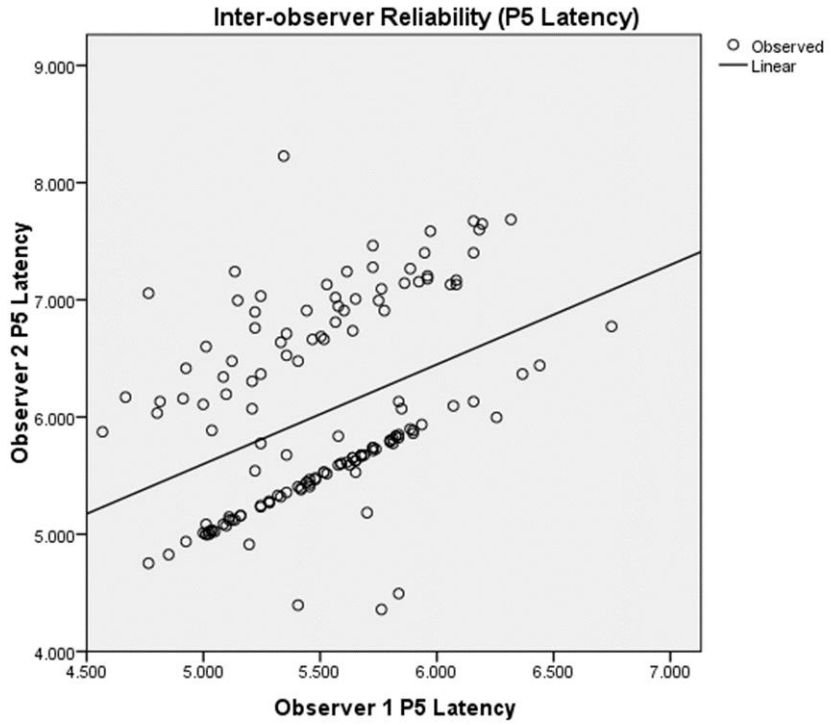


Figure 4: Scatterplot showing relationship between observer 1 and 2 in choosing P5 latency ($r^2$=.17).

**Auditory Brainstem Response Test Results**

The following figures show test results for different subgroups of mice. Results were analyzed by mouse age and mutation.

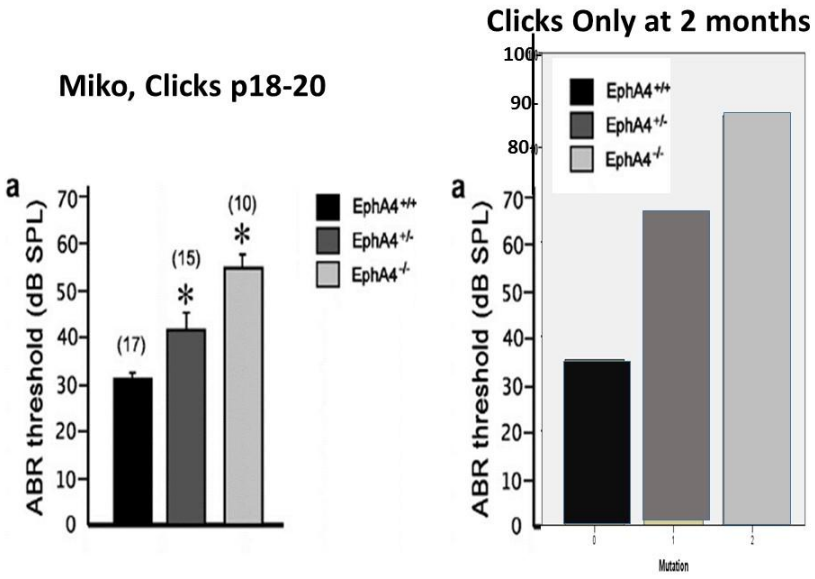


Figure 5: Bar graph comparing click ABR threshold data for the Miko et al. (2008) study and results from the current study using the 2 month old mice.

Figure 5 shows results for this study's three groups of mice compared to data from Miko et al. (2008). Mice from the Miko et al. (2008) study were between 18 to 20 days old, whereas the youngest mice from the current study were at least 2 months old. Even with this age discrepancy, the results correlated well and show an early and rapid decline in hearing. The homozygous mice do not tend to show any age related hearing loss; rather, they have congenital hearing loss that is severe starting at a young age.

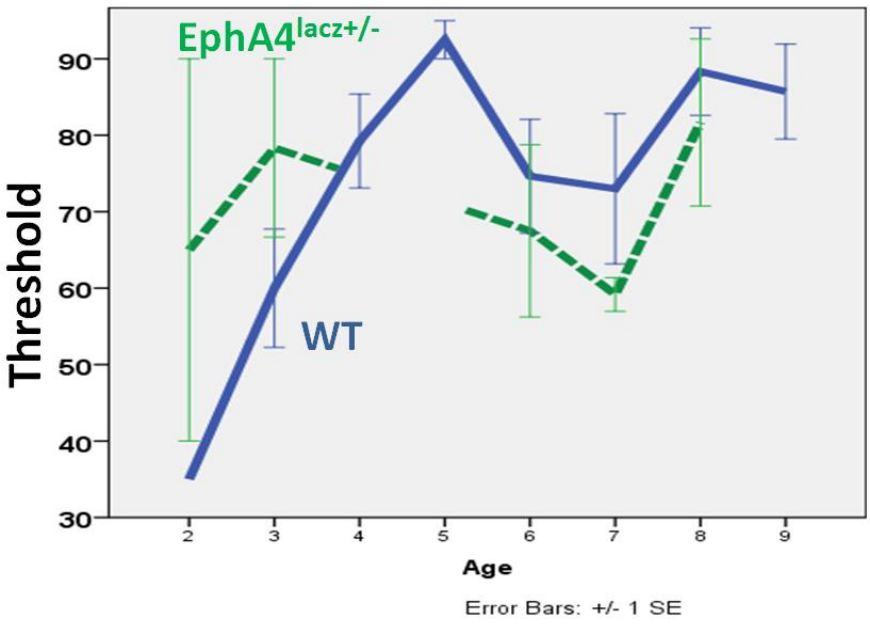


Figure 6: Line graph showing the average threshold of the wild type (solid line) mice versus the heterozygote (dotted line) mice at ages from 2-9 months old (no 4 or 5 month heterozygote mice were tested). Threshold was calculated as an average of all three stimuli (click, 8kHz tone pip, and 12 kHz tone pip). Error bars shown are for standard error.

First, a general analysis shows all thresholds (clicks, 8kHz and 12 kHz pooled) plotted over age in months for two genotypes (wild type and heterozygotes). The overall, subjective impression is that threshold for the WT mice start to increase at about 4 months of age, while the thresholds of the heterozygous mice increase later: about 8 months of age. While the threshold of the heterozygous mice might not be as good initially, the normal, age-related decline in hearing appears to be delayed.

Statistical analyses of the data in Figure 6 are challenging because about half of the data are repeated measures. The slope (threshold change over age) is 3.3 dB per month for the wild-type mice (a significant slope, $t_{128} = 4.5$, $p<.001$; $r^2=.14$). This shows that thresholds normally get worse over this time. The slope (1.4 dB/mo) for the heterozygous mutation is not significantly different from zero. This shows that,

statistically, there is no evidence of age-related hearing loss in the mutant mice. Although only one line has a significant slope, the slopes are not significantly different ($t_{166}$=1.2, p=.22).

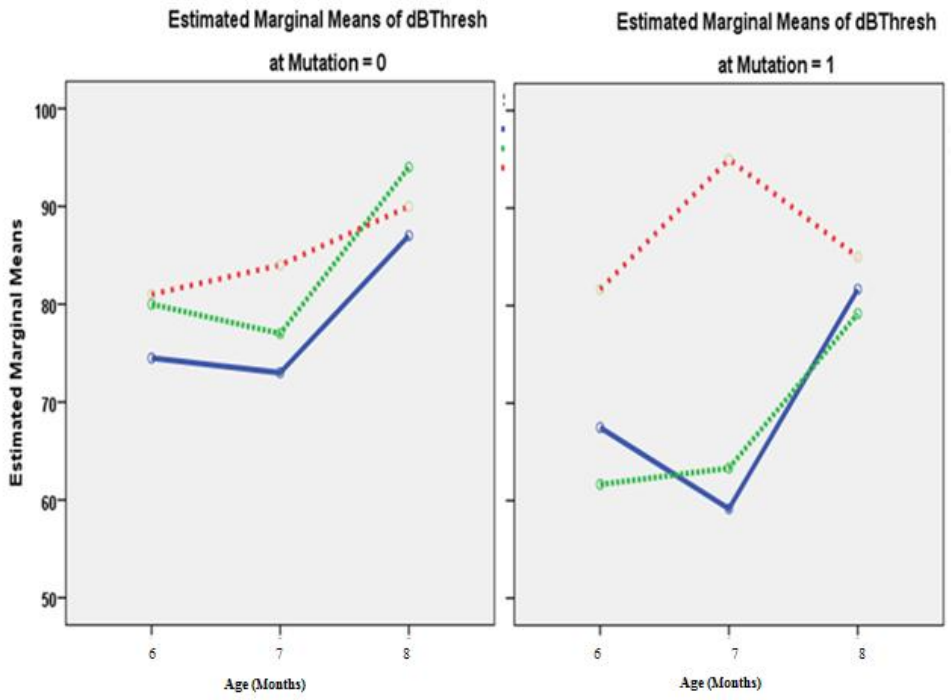


Figure 7: A line graph showing the average thresholds for the wild type mice on the left and the heterozygote mice on the right at all three time points collected in the second round of testing (at 6 months, 7 months, and 8 months of age). Click stimuli threshold is represented by the solid line in blue, 8kHz tone pip stimuli is represented by the fine dotted line in green, and 12 kHz tone pip stimuli is represented by the sparse dotted line in red.

A more rigorously appropriate analysis of this is the repeated measures at 6, 7 and 8 months of age. Repeated-measures ANOVA had 3 stimuli (clicks, 8kHz, 12 kHz) and 3 ages (6, 7 and 8 months) as within-subjects factors, and 2 genotypes (+/+ and +/-) as the between-subjects factor. There was a significant Stimulus by Mutation interaction

($F_{2,24}$>5, p=.02, $_p\eta^2$=.5) plus a stimulus by time interaction ($F_{4,24}$=5, p<.01, $_p\eta^2$=.4), both 'huge' effect sizes since $_p\eta^2$=.14 is considered 'large'. Thresholds to the different stimuli change differently over this critical time, and the mutations are differently responsive to the stimuli. The three-way interaction (Stimulus by Time by Mutation) is not significant (p=.24, $_p\eta^2$=.2 which is considered to be a 'large effect'). The main effect of Mutation is also not surprisingly not significant (p=.5, $_p\eta^2$<.1), the main effect being 'washed out' by the interactions.

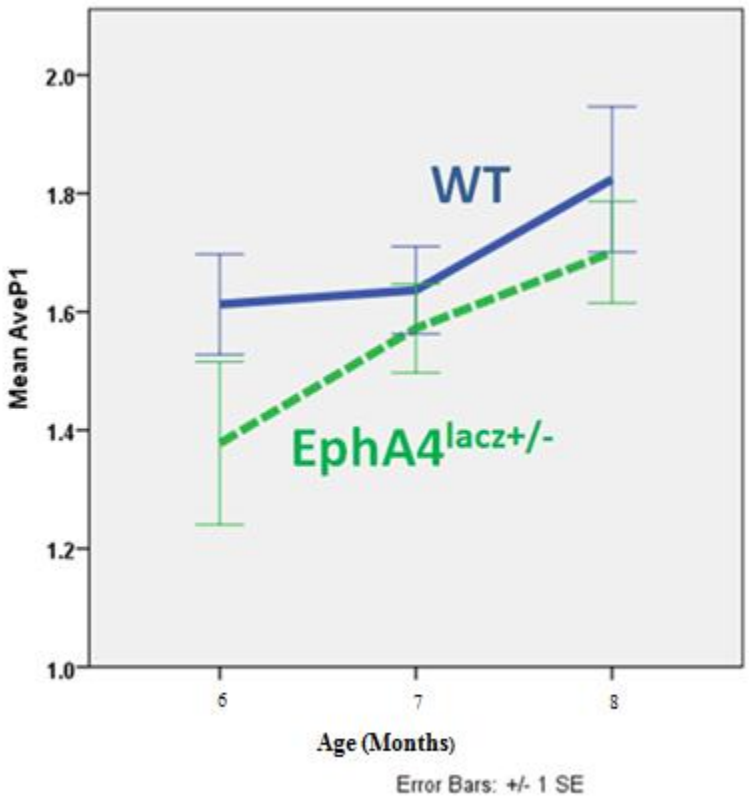


Figure 8: Average P1 latency measures in milliseconds at 90 dB for wild type (solid line) and heterozygote mice (dotted line) for the round of mice tested at 3 time points.

Because of missing values, no repeated measures ANOVA is possible for Peak 1 latencies. Pooled data seen in Figure 8 suggest no major different between the genotypes

in latencies at high stimulus intensity, although mice with the heterozygous mutation tend to have earlier P1 latencies in general.

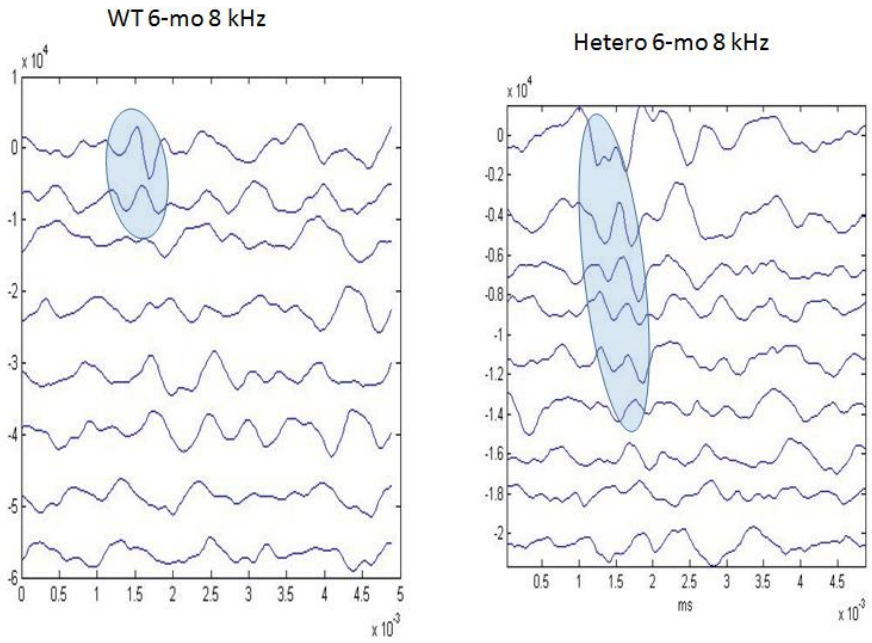


Figure 9: Averaged ABR waveforms from the combined group of 6 month old mice using an 8kHz stimulus. Highlighted areas show the progression of wave V down to threshold through decreasing intensities. It is apparent that the heterozygous mice had significantly lower thresholds than their wildtype counterparts.

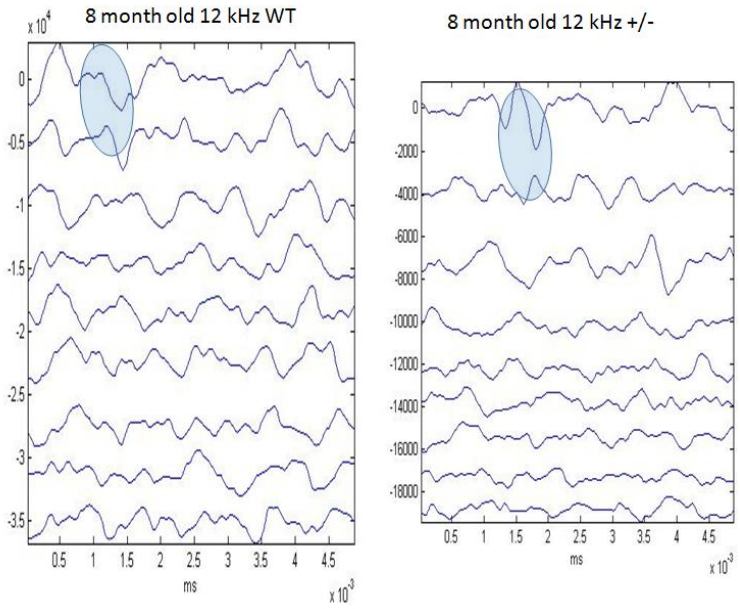


Figure 10: Averaged ABR waveforms from the combined group of 8 month old mice when tested with a 12kHz stimulus. Highlighted areas indicate the progression of wave V down to threshold through decreasing stimulus intensities. There is no significant difference in thresholds between these groups of mice when using the higher frequency stimulus.

Figures 9 and 10 depict the averaged ABR waveforms for all of the 6 and 8 month old mice from the second round of testing. These figures show that both threshold and waveform morphology were better for the heterozygous mice using the 8kHz tone pip. Both groups of mice had poor thresholds and poor waveform morphology when using the 12kHz tone pip.

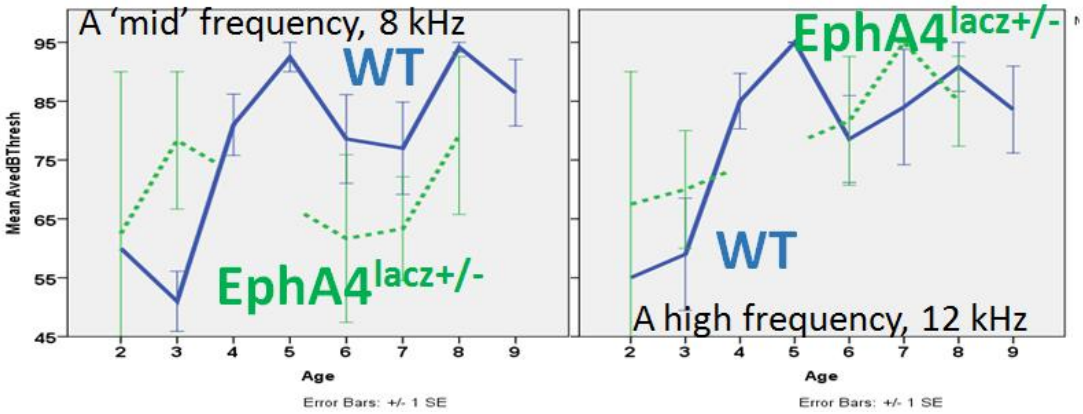


Figure 11: Line graph showing average thresholds for 8kHz and 12kHz stimuli across the age range. The solid line in blue indicates wild type mice and the dotted line in green indicates heterozygote mice.

Figure 11 presents the data for the 8kHz (mid frequency) and 12 kHz (high frequency) tone pips for a side-by-side comparison. It can be seen that heterozygote mice have generally lower thresholds for the mid frequency stimulus at later ages. When using the high frequency stimulus, both groups have similar, poor thresholds. At younger ages (2-3 months), the wild type mice have better thresholds than their heterozygote littermates when using either stimulus. When the mice reach 5-6 months of age, the wild types begin to show an increase in thresholds for both high and mid-frequency stimuli, whereas the heterozygotes tend to show an increase in thresholds for only the high frequency stimulus.

## Discussion

It is known that ABR thresholds in mice increase with increasing age. This loss is more pronounced in the higher frequencies, similar to the effect of aging on the human auditory system. Auditory brainstem response thresholds were measured in wild type (WT, +/+ or 'normal') and heterozygous (EphA4$^{lacZ/+}$ or 'half' mutant) mice. The background strain, C57BL/6J, starts losing their high-frequency hearing at about 70 days (2.5 mo) of age, so this is an ideal model to study presbycusis efficiently. The goal of the current study was to examine the effect of mutations of the EphA4 gene on the auditory aging process. The results suggest an ameliorative effect for mice with the heterozygous mutation compared to their wild type littermates. Results for mice with the homozygous mutation in the study agree with previous findings that suggest an early and rapid decrease of hearing at the beginning stages of life. Our original hypothesis that those without any mutation in EphA4 would have the best hearing was incorrect. There is some effect of having a single mutant gene that preserves middle frequency hearing for longer in these mice. It appears that having too much or too little EphA4 (the wildtypes and the homozygotes) is deleterious.

The stimulus-by-mutation interaction suggests that the altered single EphA4 allele has a different effect at different frequencies. Figure 12 below shows one conception of a possible explanation: less difference at the high frequency (12kHz) because all the mice lose their high frequency hearing first; more difference at a mid-frequency if the mutation were to delay the normally occurring age-related decline in hearing.

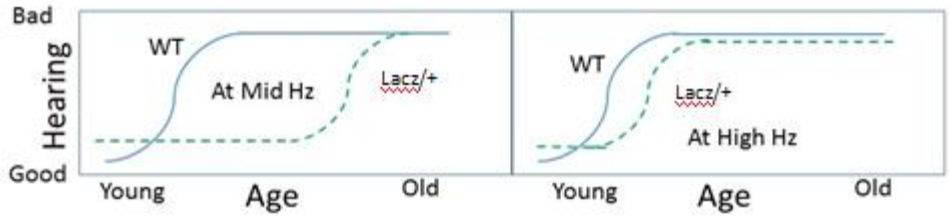


Figure 12: Possible interpretation of Figure 10- a delay in age-related hearing loss; more at mid- than-high frequencies.

There is a possibility that our research suggests another type of heterozygote advantage. This advantage can be seen in other disease processes like sickle cell anemia and cystic fibrosis. A heterozygote advantage of EphA4 was also seen in the van Hoecke et al. (2012) study that examined that effect of EphA4 mutations on zebra fish with the ALS disease process. These researchers found that the fish with a heterozygote EphA4 mutation had a greater survival rate and later age of onset for the disease. Although their study focused on the effect of EphA4 on motor neurons, it may have a similar advantageous effect on sensory neurons.

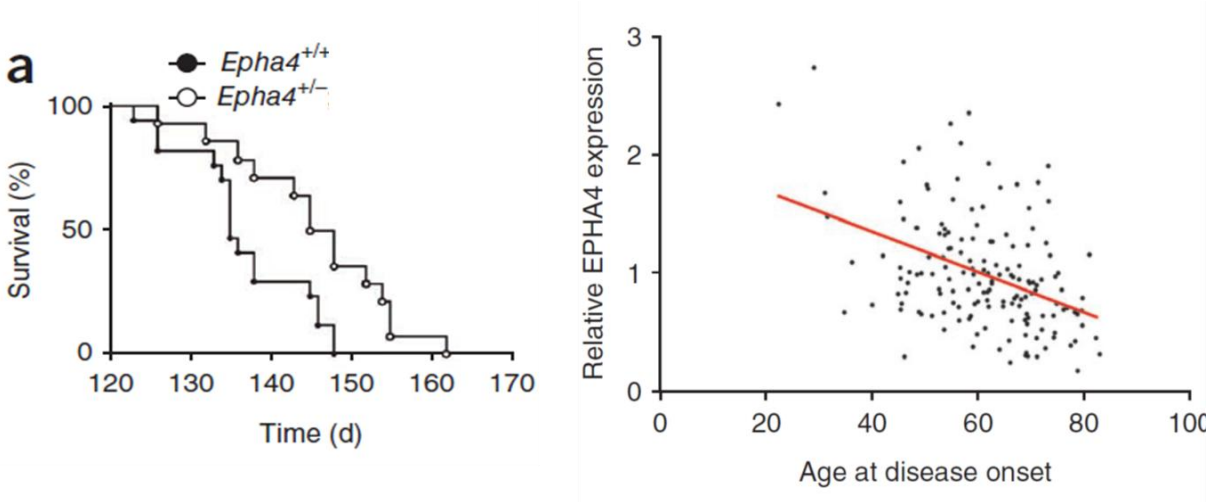


Figure 13: from van Hoecke et al. (2012) showing survival percentage over time on the left. The figure on the right shows an increase in age of onset for the disease with less relative EphA4 expression.

## Future Research

If someone could predict a drug that might affect this protein, a firmer link could be extrapolated from the seeming advantages of heterozygote expression of this protein. A continuation of this project could easily test the effects of such a drug on mice. This type of drug could potentially have wide appeal for human subjects, as hearing loss is one of the most prevalent disorders in the United States and will continue to increase with an aging population.

## Conclusions

1. There is no difference in high frequency hearing between heterozygote and wild type mice. Both groups exhibited increasing thresholds as they aged, likely due to their background strain which is used in presbycusis research.
2. There is likely a difference in mid-frequency hearing between heterozygotes and wildtypes, with the heterozygotes showing an advantage in their ABR thresholds as they age. There may be some heterozygote advantage that works to protect their hearing for this frequency region.
3. The heterozygote advantage is apparent starting at approximately 5 months of age. Previous comparison research between these two groups has been of younger mice. It appears that this advantage does not manifest until the mouse is much older.

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