Towson University

Office of Graduate Studies

Is Vulnerability to Age-Related Hearing Loss Also A Predisposition to Noise-Induced Hearing Loss?

by

Kelley Finck, B.S.

A thesis

presented to the faculty of Towson University

in partial fulfillment

of the requirements for the degree of

Clinical Doctor of Audiology

Department of Audiology, Speech Language Pathology, and Deaf Studies

Towson University

Towson, Maryland 21252

May 2018

TOWSON UNIVERSITY

OFFICE OF GRADUATE STUDIES

THESIS APPROVAL PAGE

This is to certify that the Thesis is prepared by:

Kelley Finck, B.S.

Entitled:
Is Vulnerability to Age-Related Hearing Loss Also A Predisposition for Noise-Induced Hearing Loss?

Has been approved by the thesis committee as satisfactory completing the thesis requirements for the degree: Doctor of Audiology (i.e., Doctor of Science)

A. Saradhi — 5.25.2018
Chairperson, Thesis Committee Signature Saradha Ananthakrishnan, Ph.D. Date

Ronna Hertzano — 5-23-2018
Committee Member Signature Ronna Hertzano, M.D., Ph.D. Date

Elise Smith — 5.25.18
Committee Member Signature Elise Smith, Au.D. Date

Janet V DeLany — Janet V DeLany — 5-29-18
Dean of Graduate Studies Janet DeLany Date

Acknowledgements

The completion of this project could not have been possible without the assistance of Dr. Ronna Hertzano and her research team at the University of Maryland, School of Medicine, Department of Otorhinolaryngology Head and Neck Surgery. I am grateful for the time, resources, and advice given by each and every member of the team.

I would like to thank my advisor, Dr. Saradha Ananthakrishnan, for her unending support on this project. She volunteered to take on this project with me, and I will be forever grateful for that.

Additional thanks to the remaining member of my thesis committee, Dr. Elise Smith. Her support and encouragement was so important to me during this project.

Lastly, I would like to thank my parents, Tricia and Jim Finck. Their immeasurable support has meant the world to me throughout this process. I could not have completed this project without them behind me every step of the way.

Abstract

Is Vulnerability to Age-Related Hearing Loss Also

A Predisposition to Noise-Induced Hearing Loss?

Kelley Finck, B.S.

The connections between Age-Related Hearing Loss (ARHL), Noise-Induced Hearing Loss (NIHL), and genetics remain unknown. Age-Related Hearing Loss and Noise-Induced Hearing Loss are both complex, multifactorial disorders with varied causes. Several studies have connected genetic ARHL with increased threshold shifts following a noise exposure. The aim of the study was to investigate whether heterozygous *Gfi1*$^{Cre/+}$ mice, which show an early onset ARHL, would have greater threshold shifts as compared to their wild type littermates following a noise exposure. Our results revealed that mice that were heterozygous for the *Gfi1* gene did not show increased threshold shifts as compared to their wild type littermates when exposed to loud noises. The lack of hearing threshold shifts in the *Gfi1* heterozygous mice is not similar to the pattern seen with other studies that look at effects of noise exposure on mice with a genetic predisposition to ARHL due to mutations in genes other than *Gfi1*.

This could be attributed to differences in methodology or limitations to this study. Further investigation into the *Gfi1* gene and the possible connection between ARHL and NIHL is needed.

Table of Contents

Thesis Approval Page……..ii

List of Figures……...…….vii

Introduction……..…1

Materials and Methods……...16

Results……19

Discussion……..25

Appendix A……31

Appendix B……39

Appendix C……45

References……..47

Curriculum Vitae……...55

List of Figures

Figure 1. Example of a typical NIHL audiogram. The x-axis represents frequency, and the y-axis represents intensity. X shows left ear thresholds and O shows right ear thresholds.

Figure 2. Example of a classic ARHL or presbycusis hearing loss. The x-axis represents frequency, and the y-axis represents intensity. X shows left ear thresholds and O shows right ear thresholds.

Figure 3. A visualization of genetic influences, NIHL, and ARHL. The black arrow points to the center, where all three overlap. This is the area of interest for the current study.

Figure 4. Mean absolute threshold values for male heterozygous mice (Panel A), male wild type mice (Panel B), female heterozygous mice (Panel C), and female wild type mice (Panel D). Baseline threshold values are shown (blue), as well as threshold values after noise exposure (24 hours [orange], 1 week [gray], and 3 weeks [yellow] post-noise exposure). Error bars represent one standard deviation.

Figure 5. Mean threshold shifts for male heterozygous and wild type mice at 24 hours (Panel A), 1 week (Panel B), and 3 week (Panel C) post-noise exposure for the frequencies tested (8 kHz, 16 kHz, 24 kHz, and 32 kHz). Error bars represent standard error. * $p < .05$, ** $p < .01$, *** $p < .001$

Figure 6. Mean threshold shifts for female heterozygous and wild type mice at 24 hours (Panel A), 1 week (Panel B), and 3 week (Panel C) post-noise exposure for the four frequencies tested (8 kHz, 16 kHz, 24 kHz, and 32 kHz). Error bars represent standard error.

Figure 7. Classification of hearing loss by age of onset, etiology, and language acquisition.

Figure 8. Percentage breakdown of hearing loss etiologies. Adapted from Smith et al. (2014) and Zelaya, Lucas, & Hoffman (2015).

Figure 9. Autosomal recessive inheritance pattern. A stands for a normal gene, and a stands for a mutated gene. The offspring of two carriers for an autosomal recessive mutation will be 25% unaffected, 50 % carriers or heterozygotes, and 25% affected or homozygotes.

Figure 10. Autosomal dominant inheritance pattern. A stands for an unaffected gene, and a stands for an affected gene. The offspring of an unaffected person and an affected person for an autosomal dominant disorder will be 50% unaffected and 50% affected.

Figure 11. X-linked inheritance. X stands for an unaffected female gene, x stands for an affected female gene, and Y stands for an unaffected male gene. The offspring of a cross of an unaffected male and a carrier female will have a 25% chance of being an unaffected female, a 25% chance of being a carrier female, a 25% chance of being an unaffected male, and a 25% chance of being an affected male.

Figure 12. Example of the ear notching system used and created by the lab members at University of Maryland, School of Medicine, Lab of Inner Ear Genetics.

Figure 13. An example of a PCR gel analyzed using electrophoresis techniques. Wells are number 1-9, indicating the sample being analyzed in that lane. The column on the far right, number 9, is the base pair ladder. The next two columns to the left are the wild type positive control, column 7, and *Gfi1*^{Cre} positive control, column 8. The blank column, number 6, is the negative control. The five columns farthest to the left are actual PCR products of possible mouse subjects from the current study. Lanes 1, 2, and 4 are *Gfi1*$^{Cre/+}$ heterozygous mice, and lanes 3 and 5 are *Gfi1*$^{Cre/Cre}$ homozygous mice (which were not used for the purpose of this study).

Figure 14. An example of electrode placement for the mouse subjects in this experiment. Each arrow indicates where an electrode was placed for Auditory Brainstem Response testing on the mice.

Is Vulnerability to Age-Related Hearing Loss Also a Predisposition for Noise-Induced Hearing Loss?

Forty-eight million Americans have a significant hearing loss (Blackwell, Lucas, & Clarke, 2014), with age being the strongest predictor of hearing loss for Americans age 20-69 (Hoffman, Dobie, Losonczy, Themann, & Flamme, 2016). 24.7% of individuals age 60-69 in the United States have an average hearing loss of 25 dB HL or greater in the frequencies commonly found in speech: 500, 1000, 2000, and 4000 Hz (National Health and Nutrition Examination Survey (NHANES), 2016). When several years of data from the self-reported NHANES were analyzed, hearing loss was found to be the third most prevalent health issue, directly behind heart disease and arthritis (Lin, Niparko, & Ferucci, 2011). In order to decrease the impact of the hearing loss epidemic in the United States, researchers must investigate the underlying mechanisms of hearing loss. This may lead to better treatments after the identification of hearing loss or even prophylactic treatments.

Despite the prevalence of hearing loss, the mechanisms underlying specific types of hearing loss remain unknown. For example, it is unknown why hearing loss and aging are so closely related, or how noise exposure affects the onset and severity of a hearing loss. Possible etiologies for Age Related Hearing Loss (ARHL) include carrier status for one or multiple genes that cause congenital hearing loss in the homozygous form, genetic mutations that are unrelated to early onset hearing loss, or other causes such as long term damage to inner ear structures and cognitive decline. Noise-Induced Hearing Loss

(NIHL) is damage to the auditory system from dangerous loudness levels of noise, and can be caused by many exposures to noise over time, or by a sudden, loud noise exposure. To understand these possible etiologies, one must also understand the genetics of hearing loss. For a basic tutorial on hearing loss etiologies, please see Appendix A.

Noise-Induced Hearing Loss

Noise-Induced Hearing Loss (NIHL) is a complex disorder that may stem from multiple etiologies, and can be influenced by both genetic and non-genetic factors. NIHL is damage to the auditory system from noise, which subsequently causes hearing loss. The damage can occur at many levels of the auditory system, including the hair cells, stria vascularis, neurons, and even changes in the central auditory pathways (Lynch & Kil, 2005). Due to the tonotopic organization of the cochlear sensory cells, the structures that lie in the basal end of the cochlea are the first to encounter the incoming sound waves (Von Bekesy, 1960). Therefore, the cells best tuned to respond to high frequencies, located in the base of the cochlea, are typically affected first (Von Bekesy, 1960).

NIHL is characterized by some degree of hearing loss between 3000-6000 Hz, typically creating a 'notch' in the audiogram. *Figure 1* depicts the configuration of a classic NIHL audiogram.

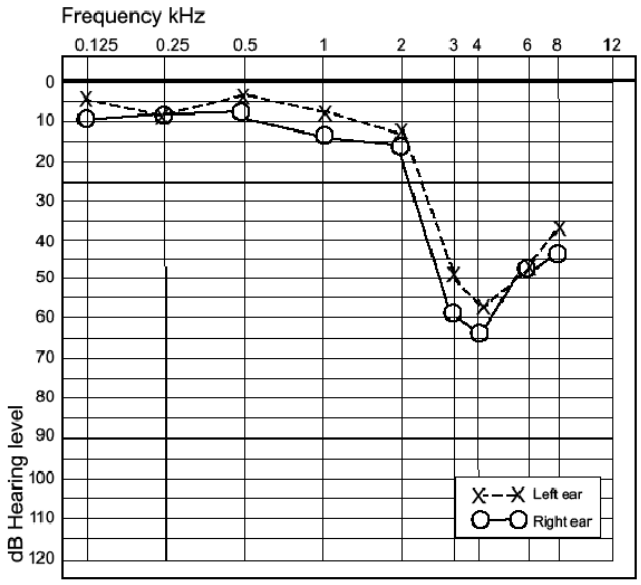

Figure 1. Example of a typical NIHL audiogram. The x-axis represents frequency, and the y-axis represents intensity. X shows left ear thresholds and O shows right ear thresholds.

This type of hearing loss can occur gradually over time, and many individuals may not notice the first signs of NIHL until it becomes more pronounced (Sliwinska-Kowalska & Davis, 2012). Typical, everyday sounds can wear down the HCs in the cochlea over many years and cause hearing loss. NIHL can also happen suddenly, when an individual is exposed to very high levels of noise for minutes or even seconds (NIOSH, 1998).

The CDC reported from the 2011-2012 NHANES that 6-24% of U.S. adults had hearing loss that may be related to noise exposure (NIOSH-CDC, 2017). More specific estimates of prevalence are difficult to obtain, as the definition of what qualifies as

hearing loss, noise exposure, and NIHL vary widely among the general population and professionals.

The National Institute for Occupational Safety and Health (NIOSH) (1998) reported that sounds above 85 dB SPL, about the loudness of heavy city traffic, can damage an individual's hearing. Examples of sounds louder than 85 dB SPL include motorcycles, any device at maximum volume played through headphones, sirens, most power tools, and firearms. Additionally, a 2011 study by Henderson, Testa, and Hartnick (2011) revealed that 17% of U.S. teenagers, age 12-19, had hearing loss. These researchers reported a concern that the number of teenagers listening to damaging levels of noise through earbuds was increasing, contributing to a slight rise in overall percentages of teenagers with hearing loss. Exposure to noise from city traffic, machinery, and music are common for many individuals, and can potentially contribute to a permanent NIHL.

The loudness level of sounds can affect how quickly the cochlea is damaged. Louder sounds damage hearing in shorter time frames than softer sounds. For example, The Center for Disease Control's (CDC's) National Institute for Occupational Safety and Health (NIOSH-CDC) reported that the recommended exposure time for 85 dB SPL sounds is 8 hours maximum per day, while any exposure time to 106 dB SPL sounds should be kept to a maximum of 3.75 minutes per day in order to avoid potential hearing loss (NIOSH, 1998). This is because for every 3 dB increase in loudness level, the sound pressure level doubles and divides the safe exposure time in half. The force of the sound pressure level of a sound is what causes traumatic damage to structures in the inner ear.

Higher sound pressure levels may damage HCs more severely, or in a shorter exposure time frame.

Interestingly, NIHL is theoretically 100% preventable, however once damage to the auditory system happens, the effects are permanent and irreversible in humans. Even though many ear structures can be damaged by noise, the most critical structures seen to deteriorate are the HCs. There are some bird and amphibian species that can spontaneously regenerate HCs, but HC regeneration does not occur in humans.

Kryter and Garinther (1965) first reported that under the same conditions, the amount of permanent hearing loss across individuals had high variability. This finding was reported as some subjects possessing "tough" ears, while others had "tender" ears, and attributed to physical differences in the ears. "Tough" ears have a lower susceptibility to NIHL, while "tender" ears have a higher susceptibility to NIHL. This was later confirmed by researchers such as Hamernik and Henderson (1974) through histological studies. Many individuals with normal hearing can be exposed to the same amount of noise for the same amount of time under the exact same conditions, and will all have unique amounts of permanent threshold shift (PTS). Many theories have been proposed as to why some individuals are more or less susceptible to NIHL following a noise exposure, including physical differences of the ear structures and genetic variability. As there is currently no way to quantify what a "tough" verses a "tender" ear looks like, research has not determined a definitive cause. This high variability in outcomes complicates the study of NIHL in humans.

Harmful noise exposure can cause temporary or permanent threshold shifts. Temporary thresholds shifts (TTS) are threshold shifts that appear after a harmful noise exposure, and will progressively resolve days or weeks after the exposure. Permanent threshold shifts (PTS) are threshold shifts from noise exposure that do not resolve, and subsequently become a permanent hearing loss. It has been thought that TTS and PTS were caused by the same breakdown in the inner ear mechanisms, but recent research has proposed a new theory.

The concept of noise-induced "hidden hearing loss" has been a recent area of research in hearing science. Hidden hearing loss is hearing loss that is not seen on a behavioral threshold audiogram. It is characterized by suprathreshold hearing difficulties in the presence of normal hearing thresholds. Kujawa and Liberman (2009) were one of the first to report that there is ongoing neural degeneration after a significant noise exposure incident in mice. Their data showed that cochlear nerve terminals present with swelling about 24-48 hours after a noise exposure. This section of auditory nerve attached to a HC, especially the inner HCs, will continue to degenerate after the swelling from the initial noise exposure has resolved. The lack of cochlear nerve regeneration will eventually result in death of the connecting inner hair cell, due to atrophy (Kujawa & Liberman, 2009). Kujawa and Liberman reported that some mice presented with normal hearing thresholds before and after the noise exposure, indicating the HCs were functioning as expected. However, the nerve still degenerated, indicating a problem with the synapse between the HC and the auditory nerve, or synaptopathy. In humans, there is presently no non-invasive technique to quantify the number of functioning synapses. Therefore, it is unknown whether noise exposure damage presents similarly in humans.

TTS after a noise exposure has been viewed as benign for many years, however this may no longer be true (Kujawa & Liberman, 2009). If this type of damage holds true in humans, this may support the idea that hearing loss is exaggerated and worsened over time when the HCs are damaged by noise exposure.

NIHL is a multifactorial disorder. The onset and severity can be affected by variables that cannot be completely controlled in the human population such as age and accidental, occupational, or recreational noise exposure. Recent research has indicated that susceptibility to permanent NIHL may also be genetically linked, and that some individuals have a predisposed susceptibility to a greater NIHL after noise exposure. Many of those genetic components have also been shown to be related to ARHL. The genetic link between NIHL and ARHL is discussed later in this paper.

Age-Related Hearing Loss

Similarly to NIHL, Age-Related Hearing Loss (ARHL) is a complex disorder that research has been attributed to multiple variables which occur and can impact an individual over time. ARHL, known as presbycusis in humans, is typically caused by a combination of many factors across the lifespan. ARHL can be caused or exacerbated by physical damage to the cochlea or auditory nerve, slower cortical functioning, NIHL, or secondary hearing loss to conditions such as high blood pressure or diabetes, and has also been shown to have a genetic connection (Fransen, Lemkens, Van Laer, & Van Camp, 2003). The genetic component of ARHL, as well as its connection to NIHL is discussed later in this paper. ARHL is characterized by a gradual loss of high frequency hearing,

which progresses to a more severe degree of hearing loss and to lower frequencies over time. *Figure 2* depicts an example audiogram of a person with presbycusis.

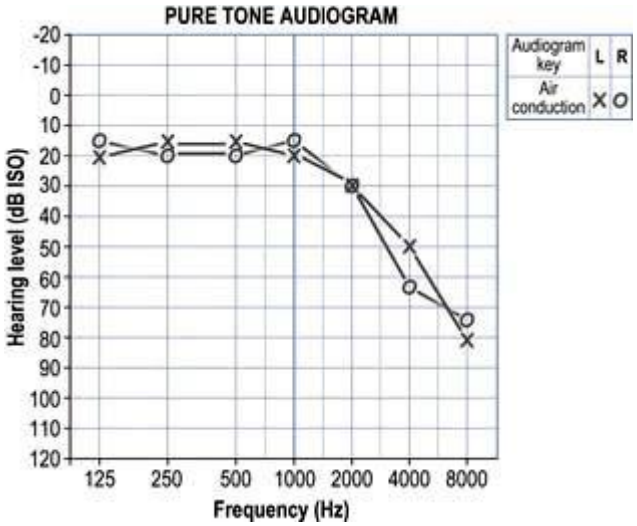

Figure 2. Example of a classic ARHL or presbycusis hearing loss. The x-axis represents frequency, and the y-axis represents intensity. X shows left ear thresholds and O shows right ear thresholds.

The age onset of an ARHL can vary greatly across individuals, but is typically seen after the fifth decade of life. In the U.S., approximately one in three individuals between age 65 and 74 have hearing loss. That number increases to half of all individuals over age 75 when considering an even older population (Cruickshanks et al., 1998). Additionally, males and females have different hearing changes related to aging. One study showed that older females had a hearing loss that progressed faster above 6000 Hz, while older males tend to have a hearing loss that progressed faster below 6000 Hz (Lee, Matthews, Dubno, & Mills, 2005).

Through a type of research study called a Genomic Wide Association Study (GWAS), researchers can study genetic variants in many different individuals to

determine if any of those genetic variants are associated with a particular trait. The first GWAS to identify a genetic locus for ARHL associated mutations in *GRM7* with an increased risk for ARHL (Friedman et al., 2009). Many other genes associated with deafness have been identified using this method. Several studies have found that if an individual is heterozygous for any of these deafness genes, they may also be predisposed to an increased risk for ARHL (Uchida, Sugiura, Sone, Ueda, & Nakashima, 2014). Deafness genes that cause hearing loss in homozygous individuals may affect heterozygous individuals later in life.

The onset and degree of variability seen across individuals with ARHL may be due to genetic factors, as well as other variables experienced over a lifetime (Kujawa & Liberman, 2006; Christensen, Frederiksen, & Hoffman, 2001).

Multifactorial Etiology of ARHL and NIHL

As discussed above, ARHL and NIHL are both complicated disorders, as each can be affected by multiple variables, both genetic and non-genetic. Genetic influences, environmental factors, noise exposure length and intensity, as well as degree and age of onset of ARHL can alter the clinical findings for an individual (Fransen et al., 2003; Kujawa & Liberman, 2006; Christensen et al., 2001). Additionally, in any given individual, ARHL and NIHL may be found together or separately. It is common to see effects of both NIHL and ARHL in a single individual (Kujawa & Liberman, 2006), rendering it complicated to tease apart the mechanisms underlying the individual disorders. It has also been shown that exposure to noise may lead to an earlier and more severe hearing loss (Kujawa & Liberman, 2006).

Research has strived to determine how ARHL, NIHL, and genetics interact in an individual. Although pieces of this question are being answered in time, a definitive connection between these three has not been discovered. *Figure 3* is a visual representation of genetic hearing loss, NIHL, and ARHL and how they interact. As shown, any of the three can occur alone, or any two of the three can interact together. For example, early exposure to noise has been shown to result in an accelerated ARHL in the mouse model, whether genetically linked or not (Erway, Shiau, Davis, & Kreig, 1996; Holme & Steele, 2004; Ohlemiller et al., 1999). The overlap of all three areas, the center of the diagram (as indicated by the black arrow), is the primary area of interest for the current study. With the high levels of occupational and recreational noise exposure common in the U.S., the growing population of aging individuals, and the increasing amount of evidence pointing towards the role of genetics in HL, understanding the interaction between genetics, NIHL, and ARHL has significant implications for public health issues. Discovering the underlying mechanisms driving NIHL and ARHL will bring society one step closer to treating or preventing these disorders.

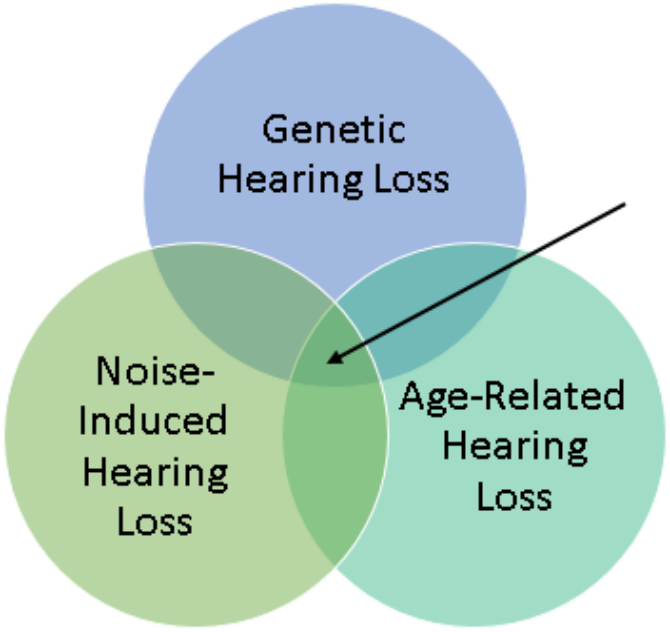

Figure 3. A visualization of genetic influences, NIHL, and ARHL. The black arrow points to the center, where all three overlap. This is the area of interest for the current study.

Human Studies on ARHL and NIHL

The effects of ARHL and NIHL have been studied in humans. In a longitudinal study of hearing in men with occupational NIHL, the NIHL influenced the degree and configuration of the ARHL seen later in life (Gates, Schmid, Kujawa, Nam, & D'Agnostino, 2000). These data suggests that ears with noise-induced damage should be considered as different from ears that have not been damaged by noise, with regard to the aging process. The cochlear neural degeneration seen long after a noise exposure may be an underlying cause to this concept. This is relevant with regard to hearing conservation

programs, and how best to treat a NIHL. Furthermore, Taylor, Pearson, and Mair (1965) reported that when corrected for age, the variability of threshold differences in female jute weavers was as high as 70 dB. These women had been exposed to constant high levels of noise in the workplace, some for decades. Yet, there was no uniformity among the degree of hearing loss revealed even when age was corrected for.

Studying the effects of ARHL and NIHL in humans is difficult for many reasons. There is inconsistency across individuals with regard to the loudness level, the type, and the duration of previous noise exposure, the use or lack of hearing protection devices, and the influences of ARHL and genetics. Additionally, the definition of hearing loss, harmful noise exposure, and hearing protection devices varies. This makes it virtually impossible to perform controlled experiments using humans, as we cannot control all of these factors over the lifetime. The inability to control and isolate the influences of variables such as length and intensity of noise exposure, genetic factors, age, and other causes of hearing loss in humans further complicates the study of the already complex interactions of ARHL and NIHL (Gates, Couropmitree, & Meyers, 1999), though these disorders have been shown to be interconnected. A common theme among all NIHL and ARHL studies is that the authors stress that these disorders are most likely the result of several factors, with many variables that are difficult to isolate even in highly controlled research studies. Due to the complexity of studying NIHL and ARHL effects in humans, researchers have turned to experimental mouse models for answers.

Mouse Studies on NIHL and ARHL

Studying complex hearing characteristics in mice was common in hearing research by the 1980s, and has continued to grow with time and technological advances (Ohlemiller et al., 2016). Research that began with phenotypic observation and rudimentary hearing estimations has expanded to include histological studies, objective auditory thresholds, and genetic/genomic analyses. Laboratory mouse models offer a shortened lifespan, plentiful and replenishable subjects, controlled genetic engineering, and can be housed in a strictly controlled environment to eliminate exposures to potentially ototoxic agents, noise, and other substances. Additionally, approximately 99% of mouse genes have human orthologs[1], allowing for the function of many genes to be translated between humans and mice (Ohlemiller et al., 2016). With the ability to isolate the effects of one variable in an experiment using the mouse model, researchers continue to pursue the identification of the underlying mechanisms of NIHL and ARHL (Kujawa & Liberman, 2006).

The Genetic Link Between ARHL and NIHL. Several studies have shown that some genetically modified mice with a predisposition for ARHL also show an increased risk for NIHL. Mutations in mouse genes such as *Cdh23* (Schwander, 2009), *Nfkb1* (Sato et al., 2006), *P2rx2* (Yan et al., 2013), and *Sodl* (McFadden, Ding, Reaume, Flood, & Salvi, 1999) have been shown to lead to ARHL and increased susceptibility to NIHL. Ohlemiller and colleagues (2016) suggest that age and noise susceptibility can often be examined together, as much of ARHL and NIHL is trauma related. This research is

[1] An ortholog is a gene found in different species that evolved from a common ancestral gene through speciation. Typically, orthologs retain the same function.

continuing to develop, and has the potential to hold information crucial to further understand human presbycusis.

Gfi1. *Gfi1* is a DNA sequence found on chromosome 1. It encodes a protein which aids in the growth and maturation of blood cells and the development of inner ear HCs (Fiolka et al., 2006). *Gfi1* has also been shown to promote cell proliferation and prevent cell death (Hertzano et al., 2004). This protein can also be found in the central nervous system, the lungs, and the retina of the eye (Fiolka et al., 2006). Researchers have studied the impact of the loss of this gene both in the phenotype and genotype of the mouse model.

In 2003, Wallis and colleagues reported that knockout mice that possess a loss of the two copies of the *Gfi1* gene (Gfi1-null) were profoundly deaf with severe balance problems. This hearing loss was reported to be caused by problems with HC development, HC organization, and HC death in the cochlea, suggesting that *Gfi1* is responsible for regulating hair cell development and organization. By inserting a cre recombinase protein[2] instead of the *Gfi1* encoding sequence, the researchers genetically engineered a model for the loss of *Gfi1*. The authors also reported that knockout mice that had only one copy of the *Gfi1* gene ($Gfi1^{cre/+}$) were indistinguishable from their wild type littermates. However, Matern and colleagues (2017) revealed through Auditory Brainstem Response (ABR) testing and histological examination of the mutant mouse inner ears that $Gfi1^{Cre/+}$ mice show an early onset progressive hearing loss. This hearing

[2] Cre recombinase is a protein used to delete segments of a target gene to create knockout mice.

loss was revealed to be significantly worse hearing than their wild type littermates at 32k Hz by 1 month of age, at 24 kHz by 3 months, and at 8k and 16k Hz by 5 months of age. The hearing loss begins at the high frequencies, and gradually progresses to lower frequencies.

Why Gfi1 in Mice is Relevant to Humans. It has been hypothesized that the gene *Pou4f3* encodes transcription factors that are responsible for the differentiation and survival of HCs in mammals (Hertzano et al., 2004). This is similar to the suggested function of *Gfi1* from Wallis et al. (2003) and Matern et al. (2017), that hair cells degenerate in the absence of Gfi1. This adds more support to the relevance of *Gfi1* in humans. *Pou4f3* underlines human autosomal dominant non-syndromic hearing loss and the progressive hearing loss associated with DFNA15 (Vahava et al., 1998; Weiss et al., 2003). *Gfi1* and *Pou4f3* have similar functions, and so may cause similar types of hearing loss in mammals.

Proposal of Experiment

Currently, it is unclear whether susceptibility to ARHL determines vulnerability to NIHL. In this proposal we investigate mice with a genetic predisposition to an accelerated ARHL and compare their susceptibility to NIHL with their wild type littermate controls. This is relevant because of the previously suggested genetic link between ARHL and NIHL. The present study aimed to determine whether mutation of the identified hearing loss gene *Gfi1* causes an increase in the susceptibility to NIHL in mice that are heterozygous for a knockout mutation of *Gfi1*, similar to other ARHL genes. Using wild-type mice and mice that are heterozygous for $Gfi1^{Cre}$ as well as both

males and females allows the researchers to examine gender effects and genetic effects. It is hypothesized that *Gfi1*$^{Cre/+}$ mice that will eventually show an ARHL will reveal a larger Permanent Threshold Shifts (PTS) when exposed to high levels of noise as compared to their wild type littermates. It may be possible to apply these results to humans who are heterozygous for mutations in genes that cause an ARHL, in that these individuals may be at a higher risk of NIHL due to their predisposition for ARHL. Knowledge of which individuals may be at risk for NIHL due to a genetic mutation can aid in the prevention or postponement of hearing loss.

Materials and Methods

Animals. The *Gfi1*^{Cre} knock-in mice were generated by Dr. Lin Gan at the University of Rochester. The animals were generously provided by Dr. Jian Zuo of the Developmental Neurobiology Department at St. Jude Children's Research Hospital for a maintained colony at the University of Maryland, Baltimore. A *Gfi1*^{Cre} colony was previously established at the University of Maryland, School of Medicine, and maintained in a C57BL/6J background. All procedures involving animals were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at the University of Maryland, Baltimore, protocol number 1015003. A total of thirty-two animals were used for this study, with the following numbers in each of the groups: 4 wild type males, 5 wild type females, 15 *Gfi1*^{Cre} heterozygous males, and 9 *Gfi1*^{Cre} heterozygous females. A total of 12 animals per group was the aim of the study, but breeding issues complicated the testing numbers. Male and female animals were evaluated separately, as recommended for all research by the National Institute of Health

(Prendergast, Onishi, & Zucker, 2014). Homozygous *Gfi1*^{Cre} animals were not used for this study, as they have no measurable hearing from birth.

Genotyping. Genotyping was performed on all mice by polymerase chain reaction (PCR) on genomic DNA extracted from identifying ear notches prior to testing (10-14 days old). Primers used for *Gfi1*^{Cre} genotyping were as follows: *Gfi1* wild type forward (5'-GGG ATA ACG GAC CAG TTG-3'), *Gfi1* wild type reverse (5'-CCG AGG GGC GTT AGG ATA-3'), *Gfi1*^{Cre} reverse (5'-GCC CAA ATG TTG CTG GAT AGT-3'). For DNA extraction: ear notches were placed in tail lysis buffer and proteinase k solution overnight in a rotisserie incubator at 56ºC. This solution was then heated to 95ºC for 10 minutes. 1 μl of tail lysis buffer with DNA was added to a solution of 1 μl of forward primer, 1 μl of wild type reverse primer, 1 μl of *Gfi1*^{Cre} reverse primer, 10 μl of Econotaq DNA Polymerase (Lucigen), and 6 μl of RNase free water. PCR stages were run in thermocycler at 95ºC, 55ºC, and 72ºC. PCR products were then visualized in a 2% agarose gel. For more information on genotyping, please see Appendix B.

Auditory Brainstem Response (ABR). ABRs were performed at several time points as a measure of hearing thresholds. First, baseline ABRs were performed at 3.5 weeks of age, and then, post-noise exposure measures were performed at 4 weeks (24 hours post-noise exposure), 5 weeks (1 week post-noise exposure), and 7 weeks of age (3 weeks post-noise exposure). For all ABR testing, mice were anesthetized using pharmaceutical grade ketamine (80-100 mg/kg) and xylazine (8-10 mg/kg) solution by intraperitoneal injection. Animals received 10 μl of ketamine-xylazine solution for every gram of weight. Approximately ten minutes were allowed after injection for the animal

to achieve total unconsciousness before testing began. Recording inverting needle electrodes were inserted posterior-inferior to the right and left auricles, a noninverting electrode needle was inserted down the midline of the skull, and a ground electrode needle was inserted at the base of the tail. In order to preserve vision, animal eye lubricant gel was applied to the animal's eyes before testing began to protect the eyes from drying out. Body temperature of the mice were maintained at a constant 37ºC by a feedback heating pad set under the animals during recording, as well as after testing in a recovery box. All recordings were performed in a sound proof box. Calibration of the speaker was performed prior to all test sessions with a measurement microphone (PCB Piezoelectronics, NY). For calibration, the recording microphone was placed ten centimeters from the speaker, in the same position as the animal. Stimuli used for testing was 2.5 millisecond condensation tone bursts, with 500 sweeps at each intensity level. Stimuli were presented bilaterally via a speaker. The speaker was situated 10 cm from the animal's ears, with the animal's nose pointing directly at the center of the speaker. Hearing thresholds were determined at 8, 16, 24, and 32 kHz using the RZ6 recording system (Tucker-Davis Technologies) for the right and left ears. Due to the two-channel recording electrode array, left and right ears were tested simultaneously and recorded separate. ABR testing began at 90 dB SPL, and stimulus intensity was decreased in 5 dB steps down to -10 dB SPL. The lowest level at which a definite ABR wave I can be detected was defined as the hearing threshold at that test frequency.

Noise Exposure. Noise exposures were performed in a sound proof box. Calibration of the speaker was confirmed prior to animals being placed in the wire chambers (dimensions of 18 X 15 X 5 cm, with 8 individual chambers measuring 4.5 X

7.5 X 5 cm in two rows of four). Up to four awake mice were placed in the center four wire chambers, which allowed for conformity of noise delivery and allowed the mice to move about freely in each separate chamber. The outside four chambers were not used for this study, as previous data obtained with this set up has shown inconsistent noise delivery to the outer four chambers, with replicable noise delivery to the central four chambers. Broadband noise with an octave band centered at 11.3 kHz (8-16 kHz) was generated through a speaker suspended in the middle of the roof of the sound proof box. Calibration of the noise was performed before every exposure with a recording microphone placed inside the wire animal cage, as close to the center of the cage as possible. The wire cage was centered beneath the speaker. Noise was presented at 102.5 dB SPL for two hours. Each noise exposure occurred at the same time of day for all animals (10:00 am).

Data and Statistical Analysis. Mean ABR threshold shifts were calculated for different groups, and t-tests were used to compare means between groups.

Results

Auditory Brainstem Response Absolute Threshold Values. ABR testing was conducted for each subject within the four groups tested (male heterozygous, male wild type, female heterozygous, and female wild type) in order to obtain auditory thresholds at baseline (no noise exposure) as well as 24 hours, 1 week, and 3 weeks post-noise exposure at 8 kHz, 16 kHz, 24 kHz, and 32 kHz. *Figure 4* depicts the mean absolute threshold values of the four groups (panel A: male heterozygous, panel B: male wild type, panel C: female heterozygous, panel D: female wild type) across all four test

sessions. Baseline measures of auditory threshold indicate that absolute thresholds are typically lowest (indicating better hearing) at 8 kHz for the BL/6 mice across all four groups tested.

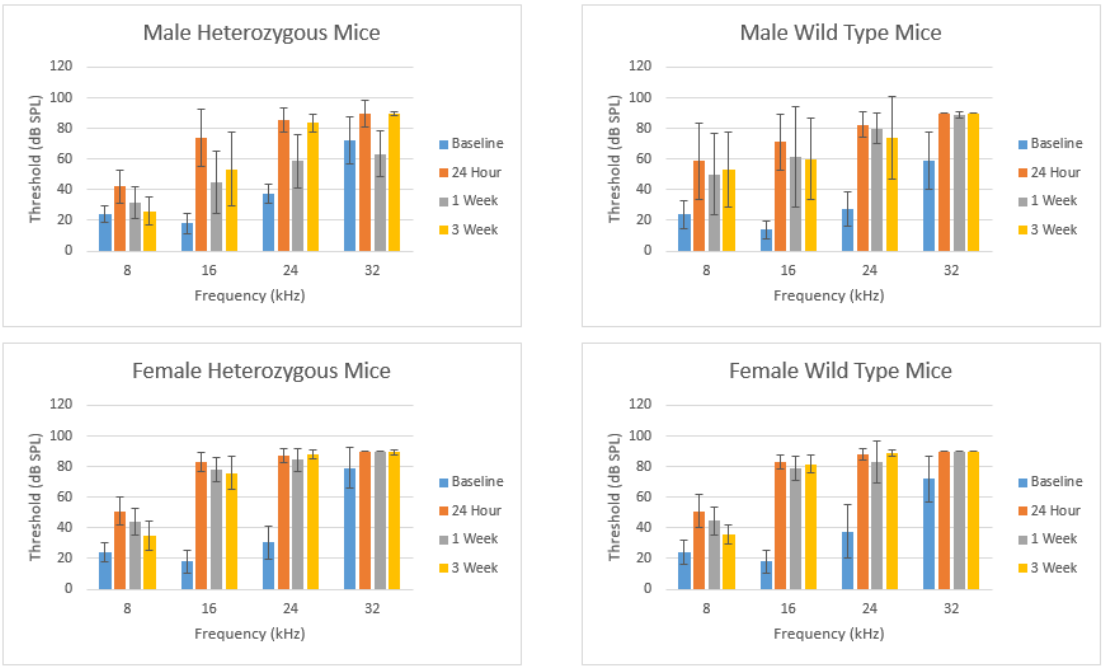

Figure 4. Mean absolute threshold values for male heterozygous mice (Panel A), male wild type mice (Panel B), female heterozygous mice (Panel C), and female wild type mice (Panel D). Baseline threshold values are shown (blue), as well as threshold values after noise exposure (24 hours [orange], 1 week [gray], and 3 weeks [yellow] post-noise exposure). Error bars represent one standard deviation.

Halfway through the data collection phase, it was discovered that the calibration microphone used to calibrate the speaker for ABR testing was potentially inaccurate. The microphone was replaced, and data collection resumed. Therefore, any differences seen

in comparisons for absolute threshold values between heterozygous and wild type mice for either gender may be confounded by skewed calibration. *Table 1* shows the mean absolute threshold values for all four groups (male heterozygous, male wild type, female heterozygous, and female wild type) with the first and second microphones analyzed separately. In general, the first microphone appeared to result in lower threshold values than the second microphone for many of the conditions. Given these observed differences in absolute threshold values obtained using the two microphones, it was decided that the effects of noise exposure on the auditory thresholds of the heterozygous and wild type mice would be better analyzed statistically using threshold *shifts* as the dependent variable as opposed to absolute thresholds.

Table 1.

Mean Threshold Values for First and Second Microphones.

		Male Heterozygous		Male Wild Type		Female Heterozygous		Female Wild Type	
		First Mic	Second Mic	First Mic	Second Mic	First Mic	Second Mic	First Mic	Second Mic
8 kHz	Baseline	20.45	25.63	16.25	31.25	17.50	26.79	15.00	26.25
	24 Hours	37.27	55.00	38.75	78.75	43.33	54.29	47.50	51.88
	1 Week	27.95	41.25	33.75	66.25	35.83	47.14	35.00	46.88
	3 Week	21.82	38.13	47.50	58.75	27.50	37.86	27.50	38.13
16 kHz	Baseline	12.50	21.25	8.75	18.75	10.00	21.43	10.00	20.00
	24 Hours	70.68	83.75	56.25	86.25	75.00	86.43	75.00	85.00
	1 Week	40.68	56.25	40.00	82.50	72.50	80.36	65.00	82.50
	3 Week	43.64	80.63	43.75	76.25	75.83	75.71	75.00	83.13
24 kHz	Baseline	26.36	50.63	17.50	37.50	18.33	35.71	20.00	41.88
	24 Hours	84.32	88.75	75.00	90.00	80.83	90.00	82.50	89.38
	1 Week	53.41	73.13	73.75	86.25	85.00	84.29	57.50	89.38
	3 Week	82.73	86.25	57.50	90.00	86.67	88.93	85.00	90.00
32 kHz	Baseline	58.64	88.13	43.75	73.75	60.83	87.14	55.00	76.25
	24 Hours	89.55	90.00	90.00	90.00	90.00	90.00	90.00	90.00
	1 Week	58.86	75.00	87.50	90.00	90.00	90.00	90.00	90.00
	3 Week	89.55	90.00	90.00	90.00	88.33	90.00	90.00	90.00

Table 1. Mean absolute threshold values for all four groups (male heterozygous, male wild type, female heterozygous, and female wild type) at the four frequencies tested (8 kHz, 16 kHz, 24 kHz, and 32 kHz) with data analyzed independently according to whether it was collected before and after the microphone change.

Auditory Brainstem Response Threshold Shifts. After consideration of the absolute values, the threshold shifts from baseline to each post-noise exposure time point were analyzed by subtracting the baseline value from the post-noise exposure value at each frequency tested (thresholds at 24 hours post-noise exposure - baseline thresholds, thresholds at 1 week post-noise exposure - baseline thresholds, and thresholds at 3 weeks post-noise exposure - baseline thresholds). *Figure 5* illustrates the 24 hour (Panel A), 1 week (Panel B), and 3 week (Panel C) post-noise exposure threshold shifts for male heterozygous and male wild type mice. Additionally, *Figure 6* illustrates the 24 hour (Panel A), 1 week (Panel B), and 3 week (Panel C) post-noise exposure threshold shifts for female heterozygous and female wild type mice. Independent t-test comparisons were used to analyze differences in the ABR threshold shifts calculated for the different time points for heterozygous and wild type mice. Male and female data were analyzed separately, as noise exposure has been known to differentially affect hearing sensitivity in male and female mice (Milon, Mitra, Song, Margulies, Casserly, Drake, … & Hertzano, 2018). Additionally, the National Institutes of Health (NIH) requires the inclusion of both male and female sexes in biological experimental measures (Clayton & Collins, 2014).

Interestingly, male wild type mice were found to have significantly higher threshold shifts as compared to male heterozygous mice at 24 kHz ($t(17) = 1.11$, $p < .05$) for the one week post-noise exposure threshold shifts, and at 8 kHz for the three week post-noise exposure threshold shifts ($t(17) = 2.38$, $p < .01$). There were no significant differences in threshold shifts found between male heterozygous and male wild type mice at any other time point and frequency. There were no significant differences in threshold

shifts for female heterozygous and female wild type mice at any of the four frequencies tested.

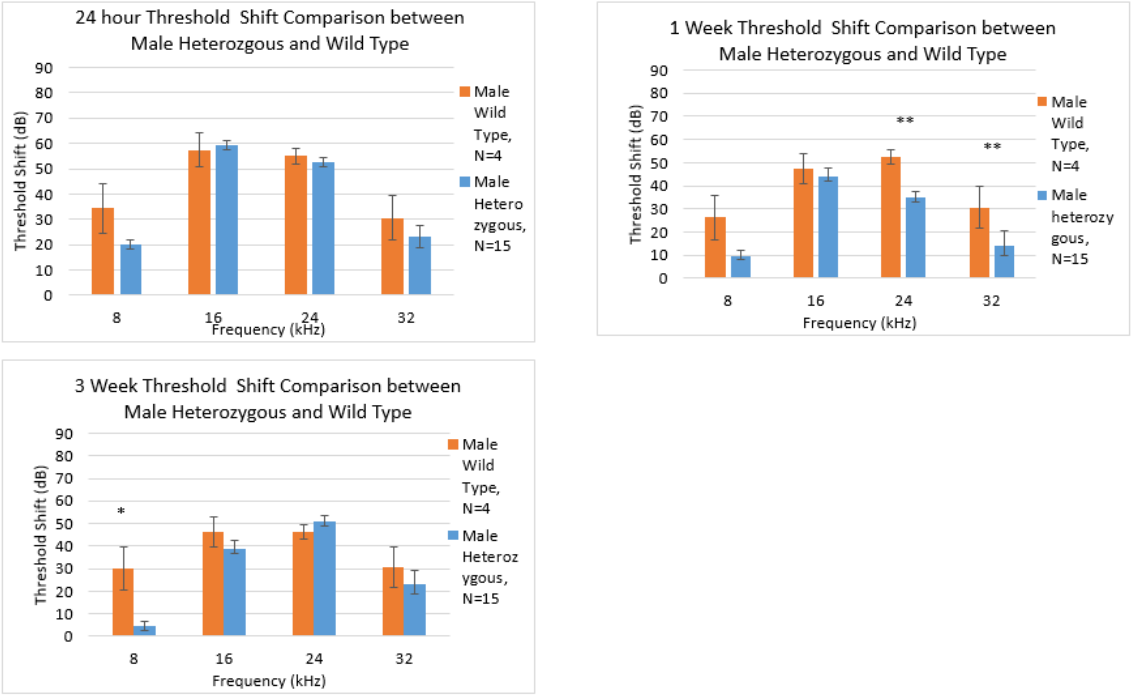

Figure 5. Mean threshold shifts for male heterozygous and wild type mice at 24 hours (Panel A), 1 week (Panel B), and 3 week (Panel C) post-noise exposure for the frequencies tested (8 kHz, 16 kHz, 24 kHz, and 32 kHz). Error bars represent standard error. * $p < .05$, ** $p < .01$, *** $p < .001$

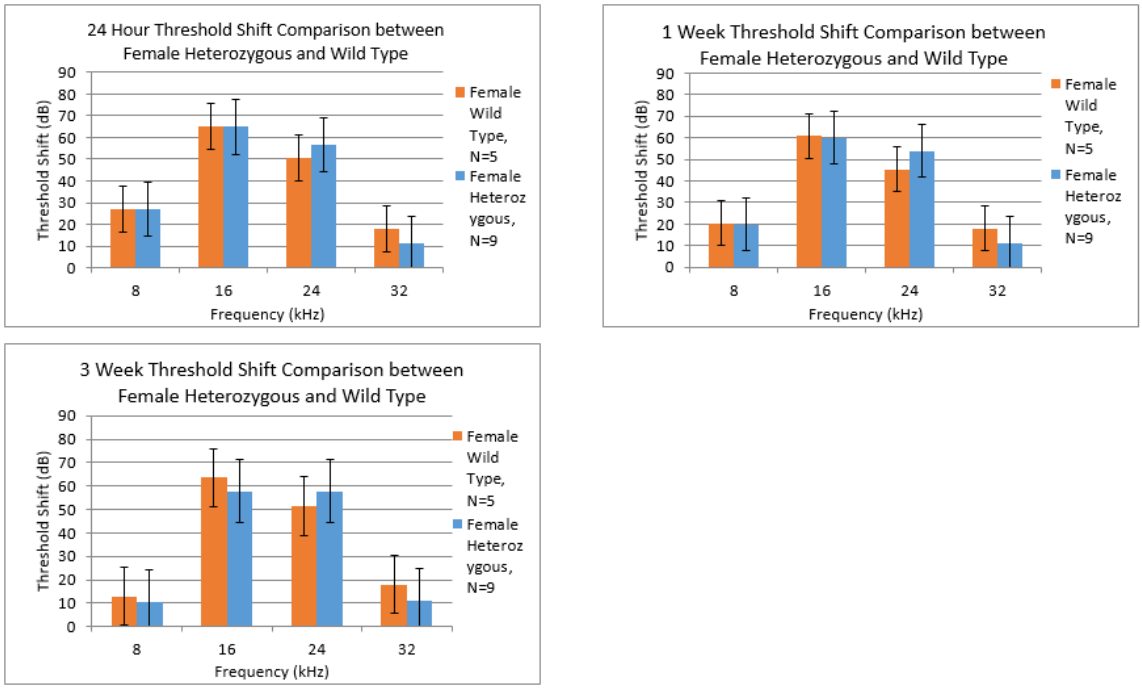

Figure 6. Mean threshold shifts for female heterozygous and wild type mice at 24 hours (Panel A), 1 week (Panel B), and 3 week (Panel C) post-noise exposure for the four frequencies tested (8 kHz, 16 kHz, 24 kHz, and 32 kHz). Error bars represent standard error.

Discussion

Multiple studies using the mouse model have found that noise exposures often result in an early onset hearing loss (Erway, Shiau, Davis, & Kreig, 1996; Holme & Steele, 2004; Ohlemiller et al., 1999). Certain mouse models with genetic mutations that are genetically predisposed to ARHL (*Cdh23, Nfkb1, P2rx2, Sodl*) have been found to be more susceptible to NIHL (Schwander, 2009; Sato et al., 2006; Yan et al., 2013; McFadden et al., 1999). Based on this prior research (Schwander, 2009; Sato et al., 2006; Yan et al., 2013; McFadden et al., 1999), it was hypothesized in the current study

that $Gfi1^{Cre/+}$ heterozygous mice, which show an early onset age-related hearing loss, would show greater threshold shifts following a noise exposure than their wild type littermates. Our findings did not indicate that $Gfi1^{Cre/+}$ mice show greater threshold shifts as compared to wild type littermates after a damaging noise exposure.

To our knowledge, no previous studies investigating the impact of noise exposure and NIHL in the *Gfi1* gene have been performed. It is possible that *Gfi1* mice which experience an early onset ARHL are not susceptible to NIHL. However, these findings are inconsistent with studies that have established susceptibility to NIHL in mice with a genetic make up that predisposes them to ARHL. This discrepancy may be due to any of the following reasons:

Methodological Differences. In the current study, baseline auditory thresholds were established for the *Gfi1* mice at three to four weeks of age, followed by post-noise exposure threshold measurements at 24 hours, one week, and three weeks. This timeline was determined based on auditory system maturation in the *Gfi1* and wild type mice, and typical durations associated with permanent threshold shifts following noise exposure. Baseline ABRs were performed at 3-4 weeks of age, at least four days before the noise exposure in order to allow the mice to have sufficient recovery time from the ABR procedure. Noise exposures were performed at four weeks of age for two reasons. First, the hearing in mice is fully developed by the time they are four weeks old. Secondly, it was the earliest the International Animal Care and Use Committee (IACUC) would allow a noise exposure to be performed. Following a noise exposure, thresholds recover exponentially with time, and reach a steady plateau around two weeks post-noise exposure (Miller et al., 1963). Post-noise exposure Auditory Brainstem Response (ABR)

tests were performed at 24 hours post-noise exposure to show the compound threshold shift (temporary threshold shift + permanent threshold shift), at one week post-noise exposure to measure the permanent threshold shifts, and at three weeks post-noise exposure to confirm the permanent threshold shift. When auditory thresholds were measured 3 weeks post noise exposure, the mice were 7 weeks old. This also ensured that any changes noticed in the mice's auditory thresholds were not confounded by age-related changes, which have been shown to occur in $Gfi1^{Cre/+}$ heterozygous mice by 3 months of age (Matern et al., 2017).

However, other studies examining the effects of noise exposure on auditory thresholds in mice with a genetic predisposition to ARHL have used varying timelines for testing. For example, one study investigated the effect of noise exposure on mouse models predisposed to ARHL. These researchers found that after controlling for the accelerated ARHL, threshold shifts continued to appear for over a year (Kujawa & Liberman, 2006). It is possible that if we found a way to control for the ARHL experienced by the $Gfi1^{Cre/+}$ mice, we could have continued to observe threshold shifts for longer than three weeks following the noise exposure.

A second methodological difference between the current study and other similar noise-exposure studies lies in the intensity of noise exposure. The current study performed noise exposures at 102.5 dB SPL for two hours. Although a specific intensity and duration of noise exposure has not been determined to be the gold standard in the research, prior research supports the use of 102.5 dB SPL noise for two hours in order to cause significant threshold shifts. Other studies examining the effects of noise exposure on auditory sensitivity have performed noise exposure at intensities ranging from 100-

110 dB SPL (Ohlemiller, Wright, & Heidbreder, 2000; Milon et al., 2018; Barden, Rellinger, Ortmann, & Ohlemiller, 2012; Erway et al., 1996; Kujawa & Liberman, 2006). A study by Erway and colleagues (1996) investigated the effect of noise exposure on four strains of mice. This study used a noise exposure intensity of 110 dB SPL, and recorded significant temporary and permanent threshold shifts in all four strains of mice (Erway et al., 1996). Moreover, a study by Lavinsky and colleagues (2015) studied the susceptibility of *Nox*^{3} heterozygous mice to NIHL as compared to their wild type littermates. An intensity of 108 dB SPL was used for these noise exposures. A significant difference between the threshold shifts of the *Nox*^{3} heterozygotes and their wild type littermates (Lavinsky et al., 2015). Although it is known that 102.5 dB SPL is an adequate intensity to induce hearing threshold shifts in mice (Ou, Bohne, and Harding, 2000), it is possible that different findings could have been seen if a louder intensity was used.

Limitations of the Study. One of the primary limitations of the study was the presence of unbalanced experimental groups and small sample sizes (male heterozygous: 15, male wild type: 4, female heterozygous: 9, female wild type: 5). Unequal sample sizes as well as small sample sizes can reduce the statistical power of the calculations, or increase the Type I Error rate, which could potentially have affected the accuracy of the threshold shift comparisons (Zimmerman, 2014). Another limitation of the study was a week long time window for baseline testing. Due to scheduling issues, baseline auditory brainstem response testing was performed anytime between age 3-4 weeks for the mice. This meant that baseline thresholds for some mice could have been obtained as early as 21 days of age or as late as 28 days of age. Ideally, to ensure uniformity, baseline

auditory thresholds should have been restricted to a narrower time window with respect to the mice's age. For example, instead of measuring baseline hearing thresholds at 21-28 days of age, they could have been restricted to day 23-24. A third limitation of this study was the change in microphones used to calibrate the speaker used for noise exposures and ABRs. Although by using the threshold shifts instead of the absolute threshold values for statistical analysis we minimized this effect, there could still have been some differences in values produced by tests when using the two microphones.

Future Research Directions. Further investigation into the *Gfi1* gene and the possible connection between ARHL and NIHL is needed. A study with increased sample sizes and equal groups would be beneficial to further determine if $Gfi1^{Cre/+}$ heterozygous mice are or are not more vulnerable to NIHL as compared to their wild type littermates. Additionally, Wave I amplitude progression analysis could be performed on the ABR data. Wave I amplitude is the sum of activity from the inner and outer hair cells, active auditory nerve fibers, functional synapses, and the endocochlear potential, with a majority of the contribution coming from the outer hair cells. Whereas Wave I amplitude is an indicator of activity at the level of the spiral ganglion, Wave I amplitude progression reflects the OHC contribution to the active processes of hearing (Milon et al., 2018). Changes in the slope of amplitude progression for any of the frequencies tested could indicate a loss of OHCs at that point on the basilar membrane, as a result of the noise exposure. Future research could also focus on immunostaining techniques to delve deeper into the mechanisms behind NIHL in this population. Immunostaining procedures allow researchers to view the Organ of Corti at the cellular level. By staining samples with different chemicals and dyes, it is possible to count the number of intact inner and

outer hair cells present in a sample. This could provide extensive information about what is happening on the cellular level in the inner ear. This is the first study, to our knowledge, that investigates the effect of noise exposure on threshold shifts in heterozygous *Gfi1*$^{Cre/+}$ mice compared to wild type littermates. Additional research is needed in this area in order to investigate the *Gfi1* gene further, and the possible connections between ARHL and NIHL.

Appendix A

Overview of Hearing Loss Etiologies

The etiology of a hearing loss can be categorized several ways. It can be categorized with regard to the age of onset of the hearing loss, whether the hearing loss began pre-lingual or post-lingual, or the etiology of the hearing loss. *Figure 5* details these categorizations. Every hearing loss can be categorized with one specific from each category. For example, a child who contracts viral meningitis at eight months old may have a pre-lingual, acquired, non-genetic hearing loss.

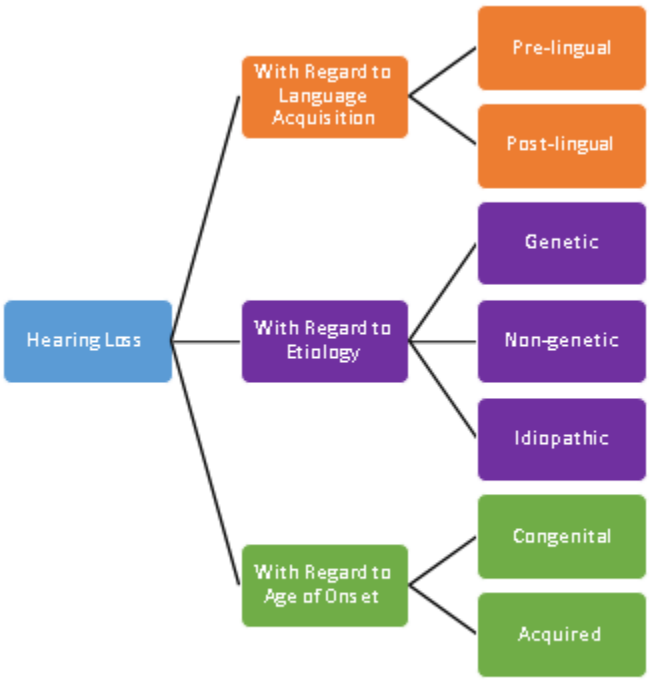

Figure 7. Classification of hearing loss by age of onset, etiology, and language acquisition.

Furthermore, the etiology of a hearing loss as genetic, non-genetic, or idiopathic hearing loss can be broken down into smaller categories. *Figure 6*, adapted from Smith, Shearer, Hildebrand, & Van Camp (2014) details the etiologies of hearing loss. Genetics can be linked to approximately 50% of hearing loss cases, while non-genetic cases and idiopathic cases each make up 25% of all cases of deafness (Smith et al., 2014).

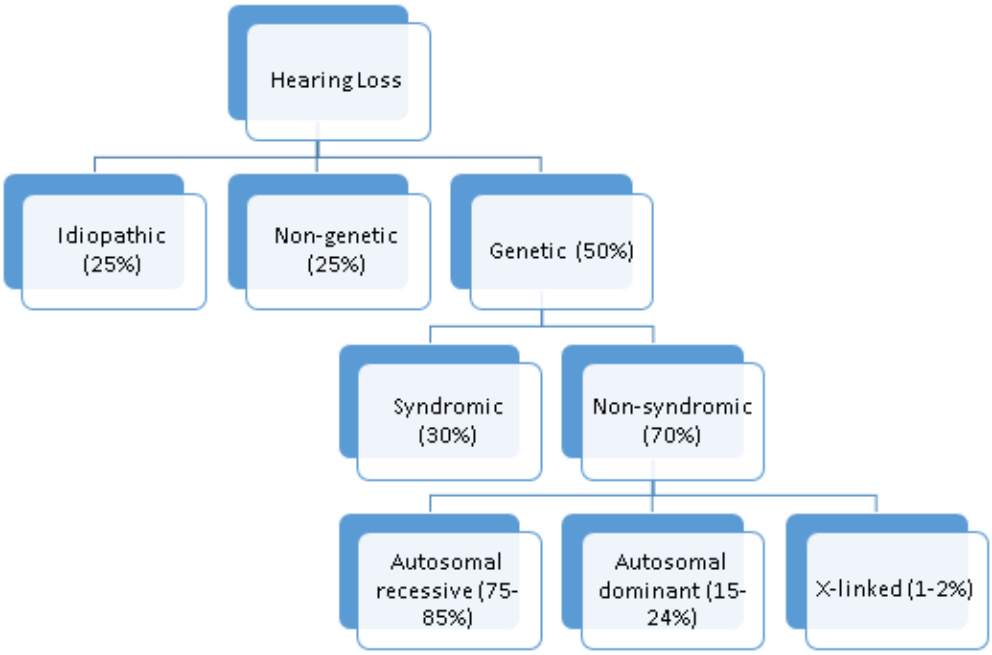

Figure 8. Percentage breakdown of hearing loss etiologies. Adapted from Smith et al. (2014) and Zelaya et al. (2015).

Genetic Hearing Loss

Additionally, cases of genetic hearing loss can be broken down into two categories: non-syndromic hearing loss (NSHL), which makes up 70% of genetic hearing

losses, and syndromic hearing loss, which makes up the remaining 30% of genetic hearing losses (Smith et al., 2014).

Non-syndromic hearing loss. NSHL is hearing loss that is not associated with any other systemic dysfunction. These genetic mutations can be inherited via autosomal inheritance, meaning the genes are found on any of the 22 human autosomal chromosome pairs, or can be inherited by transmission of a mutation on a sex chromosome, typically the X chromosome (NIH, 2017). Non-syndromic genetic hearing loss (NSHL) can be categorized by its inheritance pattern into three categories: autosomal recessive NSHL, autosomal dominant NSHL, and X-linked NSHL.

Autosomal recessive inheritance. A clear majority of all NSHL, approximately 75-85% of cases, are inherited in an autosomal recessive fashion (Smith et al., 2014). Genes inherited in an autosomal recessive pattern will be expressed only if the individual possesses two mutated copies of the gene. Individuals with only one copy of the gene are designated as carriers or heterozygotes. Carriers do not express the symptoms of the mutated gene, but can potentially pass down the mutated gene to their offspring. *Figure 7* depicts the cross of two individuals who are heterozygous, or have one unaffected and one mutated gene, for an autosomal recessive disorder. Every offspring will have a 25% chance of being unaffected, a 25% chance of being affected, and a 50% chance of being a carrier for the disorder. Human autosomal recessive NSHL genes that are identified are designated by the letters DFNB (NIH, 2017). It has been estimated that 50% of all autosomal recessive NSHL can be attributed to a number of different mutations on the DFNB1 locus (Smith et al., 2014). Some examples of identified autosomal recessive deafness loci are DFNB1, which causes a prelingual hearing loss, and DFNB9 and

DFNB12, both of which cause a severe to profound prelingual hearing loss (Van Camp & Smith, 2010).

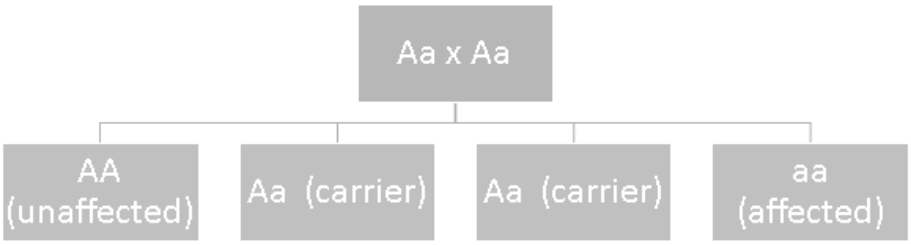

Figure 9. Autosomal recessive inheritance pattern. A stands for a normal gene, and a stands for a mutated gene. The offspring of two carriers for an autosomal recessive mutation will be 25% unaffected, 50 % carriers or heterozygotes, and 25% affected or homozygotes.

Autosomal dominant inheritance. A less common inheritance pattern for NSHL is autosomal dominant inheritance. Smith and colleagues (2014) estimated that 15-24% of all NSHL is inherited by autosomal dominant inheritance. Individuals with an autosomal dominant mutation require only one mutated copy of the gene to express the disorder. Thus, unlike with autosomal recessive inheritance, heterozygotes for an autosomal dominant mutation will express the effects of the mutation, even though they hold one normal copy of the gene. *Figure 8* depicts the cross of an affected person with an autosomal dominant disorder and an unaffected person. The offspring of an unaffected person and an affected person for an autosomal dominant disorder will be

50% unaffected and 50% affected. Human autosomal dominant NSHL mutations that have been identified are designated by the letters DFNA (NIH, 2017). Examples of autosomal dominant deafness loci are DFNA3, which causes a prelingual, progressive, high frequency hearing loss, and DFNA2, which causes a post lingual, progressive, high frequency hearing loss in the second decade of life (Van Camp & Smith, 2010).

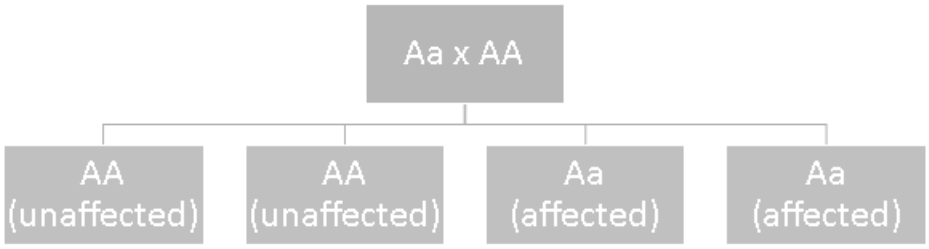

Figure 10. Autosomal dominant inheritance pattern. A stands for an unaffected gene, and a stands for an affected gene. The offspring of an unaffected person and an affected person for an autosomal dominant disorder will be 50% unaffected and 50% affected.

X-linked inheritance. A rare form of NSHL inheritance, X-linked inheritance, occurs in approximately 1-2% of all NSHL cases (Smith et al., 2014). X-linked mutations are inherited on the X chromosome. Males will express an inherited X-linked mutation, while females are typically carriers for the mutation. Heterozygote females for an X-linked mutation may or may not show the effects of the mutation, and there is a high degree of variability in this regard depending on the mutation in question. Families with an X-linked mutation often see the disorder 'skip' a generation, as it is passed from

a grandfather to a mother to a son, where only the grandfather and son express the disorder, and the mother is a carrier. *Figure 9* depicts the offspring of an unaffected male and a carrier female. The offspring of a cross of an unaffected male and a carrier female will have a 25% chance of being an unaffected female, a 25% chance of being a carrier female, a 25% chance of being an unaffected male, and a 25% chance of being an affected male. NSHL mutations that are of x-linked inheritance are designated by the letters DFNX (NIH, 2017). Some examples of X-linked deafness loci are DFNX1, which causes a severe to profound post-lingual, progressive, sensorineural hearing loss and DFNX2, which causes a pre-lingual, progressive, mixed hearing loss (Van Camp & Smith, 2010).

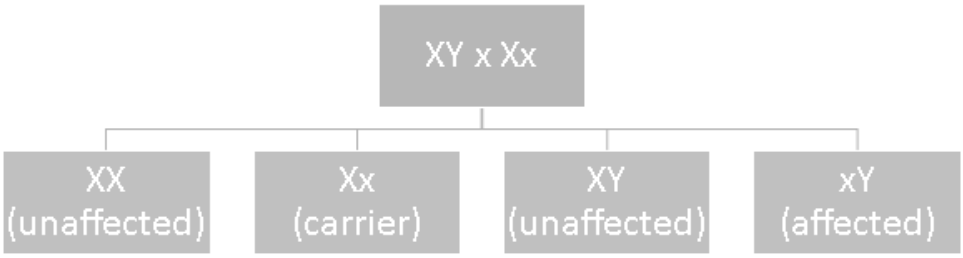

Figure 11. X-linked inheritance. X stands for an unaffected female gene, x stands for an affected female gene, and Y stands for an unaffected male gene. The offspring of a cross of an unaffected male and a carrier female will have a 25% chance of being an unaffected female, a 25% chance of being a carrier female, a 25% chance of being an unaffected male, and a 25% chance of being an affected male.

According to the HUGO Gene Nomenclature Committee (2017), there are currently 56 DFNA gene loci identified, 91 DFNB gene loci identified, and 7 DFNX gene loci identified, for a total of 154 identified human deafness related genes.

Syndromic hearing loss. Syndromes are identifiable patterns or constellations of symptoms. Over 400 syndromes which include hearing loss as a symptom have been identified. Syndromic hearing losses are genetically based in nature, but can be inherited or spontaneous. Much like NSHL, syndromic hearing loss is inherited in autosomal recessive, autosomal dominant, or X-linked fashion (Toriello, Reardon, & Gorlin, 2004). Syndromes associated with hearing loss include Waardenburg syndrome, Down syndrome, Pendred syndrome, and Usher syndrome (Smith et al., 2014).

Non-Genetic Hearing Loss

Non-genetic hearing losses can be attributed to pharmaceutical, pathological, or traumatic causes. There are many causes of non-genetic hearing loss including viruses, ototoxicity, trauma to the outer, middle, or inner ear, middle ear infections, and hearing loss secondary to other diseases (Hearing Loss Association of America). Some causes of non-genetic hearing loss, such as otosclerosis or a tympanic membrane perforation, can be fixed with surgical intervention. Other causes of non-genetic hearing loss may be permanent, such as ototoxicity.

Idiopathic Hearing Loss

Idiopathic causes are hearing loss without a known cause. Despite the development of technological advances and increased knowledge of the underlying mechanisms of hearing loss, there are still many cases of hearing loss which cannot be attributed to a specific cause (Smith et al., 2014).

Appendix B

Terms and Concepts:

Genotyping: Process of determining the genetic makeup of a DNA sample.

Ear notching: Mice have ear punches performed on them for two reasons. One reason is as an identifying mark, as the location and number of punches relates to a numbered key. Another reason is to obtain a DNA sample to be used in the genotyping process. Below is a copy of the notching system used for this study, created by lab members at the University of Maryland, School of Medicine, Lab of Inner Ear Genetics.

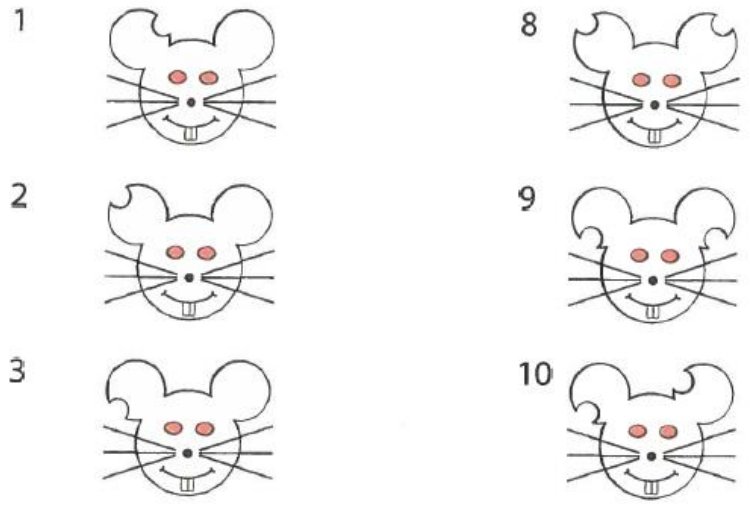

Figure 12. Example of the ear notching system used and created by the lab members at University of Maryland, School of Medicine, Lab of Inner Ear Genetics.

Polymerase Chain Reaction (PCR): process used to amplify or copy a single piece or several pieces of DNA. PCRs are run with all three of the following steps multiple times to get many replicated strands of the target DNA.

Steps in a PCR:

1. Denaturing- the double helix of DNA strands separates. This is done at around 95ºC.

2. Annealing- the primer strands attach to their complementary sequences on a single helix strand of DNA. This step is done at around 50ºC.

3. Extending- DNA polymerase is activated, and begins to copy the DNA single strand using the free floating nucleotide bases in the solution.

Primer: A single helix strand of DNA that is typically 18-22 base pairs. It will attach itself to sites on the DNA strand that correspond with its specific sequence of base pairs. Primers can be designed to match any DNA sequence. This will be the starting point for the amplification/copying process. DNA polymerase needs existing DNA to begin replication of a segment, and the primers give it a starting location. Typically, a minimum of two primers are used, one for the beginning of the target sequence and one for the end of the target sequence to confirm the gene in both directions. For this study, a total of three primers were used. The forward primer will attach to the target sequence for all mice. The wild type reverse primer and/or the *Gfi1*^{Cre} reverse primer will attach to end of the target sequence. The length of the strand created between the two primers is how the genotype is identified.

Electrophoresis: Process by which PCR products are visualized. This uses a 2% agarose gel with small wells for the PCR products to be inserted into. Opposite sides of the gel are then polarized. DNA is inherently negatively charged, and will travel through the gel towards the positively polarized side. Smaller strands will travel faster and farther than longer strands. With the use of an example DNA base pair ladder beside the PCR products, the approximate length of the DNA samples can be determined. Negative and/or positive controls can also be used as a known guide for easier determination of genotype. Below is an example of a gel after electrophoresis from this study. The column on the far right, number 9, is the base pair ladder. Within the ladder, the brighter band slightly above the bands of the positive control represents 1000 base pairs, and the brighter band slightly below the bands of the positive controls represents 500 base pairs. The next two columns to the left are the wild type positive control, column 7, and *Gfi1*^{Cre} positive control, column 8. The blank column, number 6, is the negative control. The five columns farthest to the left are actual PCR products of possible mouse subjects from the current study. When compared to columns 7 and 8, it is clear that column 1, 2, and 4, have both the wild type and *Gfi1*^{Cre} gene, and so are *Gfi1*^{Cre} heterozygous mice (*Gfi1*$^{Cre/+}$). Columns 3 and 5 show only the *Gfi1*^{Cre} gene, and therefore are *Gfi1*^{Cre}

homozygous mice (*Gfi1*$^{Cre/Cre}$). Animals 1, 2, and 4 were used as part of this study.

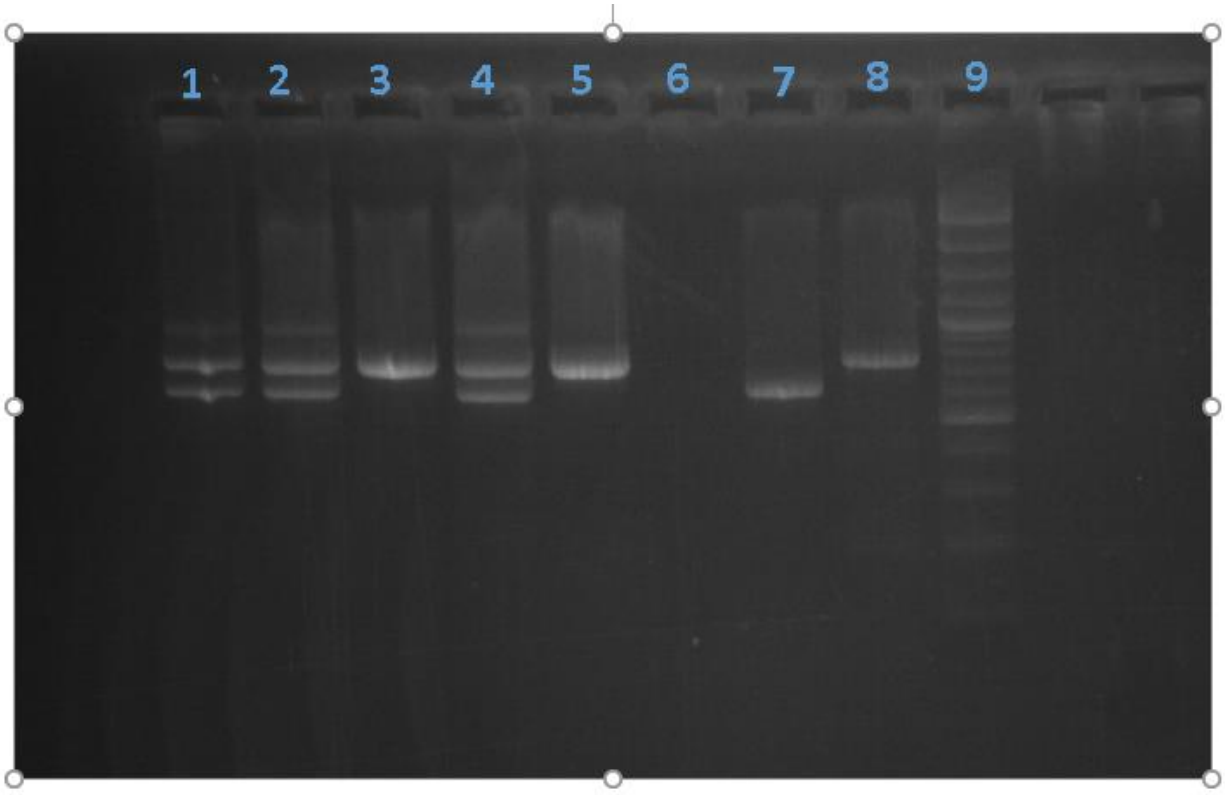

Figure 13. An example of a PCR gel analyzed using electrophoresis techniques. Wells are number 1-9, indicating the sample being analyzed in that lane. The column on the far right, number 9, is the base pair ladder. The next two columns to the left are the wild type positive control, column 7, and *Gfi1*^{Cre} positive control, column 8. The blank column, number 6, is the negative control. The five columns farthest to the left are actual PCR products of possible mouse subjects from the current study. Lanes 1, 2, and 4 are *Gfi1*$^{Cre/+}$ heterozygous mice, and lanes 3 and 5 are *Gfi1*$^{Cre/Cre}$ homozygous mice (which were not used for the purpose of this study).

Electrode placement sites: Recording inverting needle electrodes will be inserted posterior-inferior to the right and left auricles, a noninverting electrode needle will be

inserted down the midline of the skull, and a ground electrode needle will be inserted at the base of the tail. An example of electrode placement can be found in the picture below.

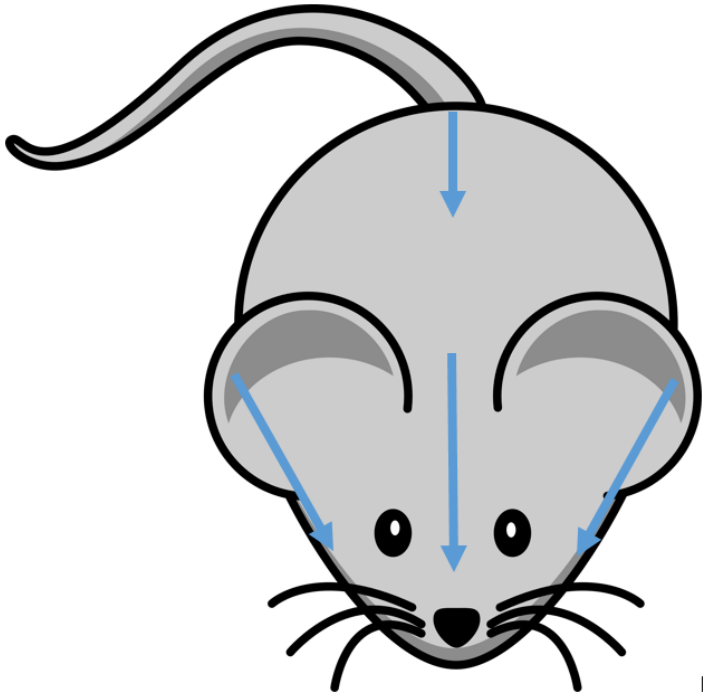

Figure 14. An example of electrode placement for the mouse subjects in this experiment. Each arrow indicates where an electrode was placed for Auditory Brainstem Response testing on the mice.

Solutions:

Tail lysis buffer and proteinase k: tail lysis buffer begins to digest the DNA for the PCR process. Proteinase k decontaminates the sample by digesting any contaminates present in the sample.

PCR solution: 1 μl of each of the three primers. Econotaq includes the DNA polymerase, nucleotide bases, as well as green tracking dye. 1 μl of the DNA sample is added. The solution is finished with RNase free water (purified water).

Appendix C

UNIVERSITY OF MARYLAND
SCHOOL OF MEDICINE
Office of Animal Welfare Assurance

655 W. Baltimore Street
BRB, Mezzanine Ste. M023
Baltimore, MD 21201-1559

email: iacuc@som.umaryland.edu
voice: (410) 706-7859/8470
Assurance Number: A3200-01

DATE: January 30, 2017

TO: Ronna Hertzano, M.D., Ph.D.
Department of Otorhinolaryngology
16 S Eutaw St. Suite 500

FROM: Institutional Animal Care and Use Committee

RE: IACUC Protocol #1015003
"A Cell-Type Specific Approach to Study Auditory and Vestibular Function"

This is to certify that the Institutional Animal Care and Use Committee APPROVED your amendment dated January 23, 2017 via the DESIGNATED review process.

Please note that your protocol will expire on October 16, 2018.

If you have any questions, please do not hesitate to contact the Office of Animal Welfare Assurance by email (iacuc@som.umaryland.edu) or by phone (706-7859 / 8470).

John B. Sacci, Jr., Ph.D.
IACUC Chair

UNIVERSITY OF MARYLAND
SCHOOL OF MEDICINE
Office of Animal Welfare Assurance

655 W. Baltimore Street
BRB, Mezzanine Ste. M023
Baltimore, MD 21201-1559

email: iacuc@som.umaryland.edu
voice: (410) 706-7859 / 8470
Assurance Number: A3200-01

DATE: March 27, 2018

TO: Ronna Hertzano, M.D., Ph.D.
Department of Otorhinolaryngology
16 S Eutaw St. Suite 500

FROM: Institutional Animal Care and Use Committee

RE: IACUC PROTOCOL #1015003
"A Cell-Type Specific Approach to Study Auditory and Vestibular Function"

The Institutional Animal Care and Use Committee reviewed and APPROVED your amendment dated March 23, 2018.

Please note that the expiration date for this protocol is October 16, 2018.

If you have any questions, please do not hesitate to contact the Office of Animal Welfare Assurance by email (iacuc@som.umaryland.edu) or by phone (at 706-7859 / 8470).

John B. Sacci, Jr., Ph.D.
IACUC Chair

References

Barden, E. K., Rellinger, E. A., Ortmann, A. J., & Ohlemiller, K. K. (2012). Inheritance patterns of noise vulnerability and "protectability" in (C57BL/6J x CBA/J) F1 hybrid mice. *Journal of the American Academy of Audiology, 23*, 332-340. doi: 10.3766/jaaa.23.5.4

Blackwell, D. L., Lucas, J. W., & Clarke, T. C. (2014). Summary health statistics for U.S. adults: National health interview survey. *Vital Health Statistics, 10*(260).

Christensen, K., Frederiksen, H., & Hoffman, H. J. (2001). Genetic and environmental influences on self-reported hearing in the old and oldest old. *Journal of the American Geriatrics Society, 49*(11), 1512-1517. doi: 10.1046/j.1532-5415.2001.4911245.x

Clayton, J.A., and Collins, F.S. (2014). Policy: NIH to balance sex in cell and animal studies. *Nature*, *509*, 282–3.

Cruickshanks, K. J., Wiley, T. L., Tweed, T. S., Klein, B. E., Klein R., Mares-Perlman, J. A., & Nondahl, D. M. (1998). Prevalence of hearing loss in older adults in Beaver Dam, Wisconsin. The epidemiology of hearing loss study. *American Journal of Epidemiology, 148*(9), 879-886.

Erway, L. C., Shiau, Y., Davis, R. R., & Krieg, E. F. (1996). Genetics of age-related hearing loss in mice. III. Susceptibility of inbred and F1 hybrid strains to noise-induced hearing loss. *Hearing Research, 93*, 181-187.

Fiolka, K., Hertzano, R., Vassen, L., Zeng, H., Hermesh, O., Avraham, K. B., … Moroy, T. (2006). Gfi1 and Gfi1b act equivalently in haematopoiesis, but have distinct, non-overlapping functions in inner ear development. *Scientific Report, 7*(3), 326-333.

Fransen, E., Lemkens, N., Van Laer, L., & Van Camp, G. (2003). Age-related hearing impairment (ARHI): Environmental risk factors and genetic prospects. *Experimental Gerontology*, *38*(4), 353-359. doi: 10.1016/S0531-5565(03)00032-9

Friedman, R. A., Van Laer, L., Huentelman, M. J., Sheth, S. S., Van Eyken, E., Corneveaux, J. J., … & Van Camp, G. (2009). GRM7 variants confer susceptibility to age-related hearing impairment. *Human Molecular Genetics, 18*(4), 785-796. doi: https://doi.org/10.1093/hmg/ddn402

Gates, G. A., Couropmitree, N. N., Meyers, R. H. (1999). Genetic associations in age-related hearing thresholds. *Archives of Otolaryngology, Head and Neck Surgery, 125*(6), 654-659. doi: 10.1001/archotol.125.6.654

Gates, G. A., Schmid, P., Kujawa, S. G., Nam, B., & D'Agostino, R. (2000). Longitudinal threshold changes in older men with audiometric notches. *Hearing Research, 141*(1-2), 220-228. doi: 10.1016/S0378-5955(99)002233

Hamernik, R. P., & Henderson, D. (1974). Impulse noise trauma: A study of histological susceptibility. *Archives of Otolaryngology, 99*, 118-121.

Hearing Loss Association of America. Types, causes, and treatments. Retrieved from http://www.hearingloss.org/content/types-causes-and-treatment

Henderson, E., Testa, M. A., & Hartnick, C. (2011). Prevalence of noise-induced hearing-threshold shifts and hearing loss among US youths. *Pediatrics, 127*(1). doi: 10.1542/peds.2010-0926

Hertzano, R., Montcouquiol, M., Rashi-Elkeles, S., Elkon, R., Yücel, R., Frankel, W. N., & ... Avraham, K. B. (2004). Transcription profiling of inner ears from Pou4f3ddl/ddl identifies Gfi1 as a target of the Pou4f3 deafness gene. *Human Molecular Genetics, 13*(18), 2143.

Hoffman, H. J., Dobie, R. A., Losonczy, K. G., Themann, C. L., Flamme, G. A. (2016). Declining prevalence of hearing loss in US adults aged 20 to 69 years. *Journal of the American Medical Association, Otolaryngology – Head & Neck Surgery.*

Holme, R. H., & Steel, K. P. (2004) Progressive hearing loss and increased susceptibility to noise-induced hearing loss in mice carrying a Cdh23 but not a Myo7a mutation. *Journal of the Association of Research in Otolaryngology, 5,* 66-79.

HUGO Gene Nomenclature Committee. (2017). Gene family: Deafness associated genes. Retrieved from http://www.genenames.org/cgi-bin/genefamilies/set/1152

Kryter, K. D., & Garinther, G. R. (1965). Auditory effects of acoustic impulses from firearms. *Academy of Otolaryngology, 211,* 1-20.

Kujawa, S. G., & Liberman, M. C. (2006). Acceleration of age-related hearing loss by early noise exposure: Evidence of a misspent youth. *The Journal of Neuroscience, 26*(7), 2115-2123. doi: 10.1523/JNEUROSCI.4985-05.2006

Kujawa, S. G., & Liberman, M. C. (2009). Adding insult to injury: Cochlear nerve degeneration after "temporary" noise-induced hearing loss. *Journal of Neuroscience, 29*(45), 14077-14085. doi: 10.1523/JNEUROSCI.2845-09.2009

Lee, F. S., Matthews, L. J., Dubno, J. R., & Mills, J. H. (2005). Longitudinal study of pure-tone thresholds in older persons. *Ear and Hearing, 26*(1), 1-11.

Lin, F. R., Niparko, J. K., & Ferrucci, L. (2011). Hearing loss prevalence in the United States. *Archives of Internal Medicine, 171*(20), 1851-1853. doi:10.1001/archinternmed.2011.506

Lynch, E. D., & Kil, J. (2005). Compounds for the prevention and treatment of noise-induced hearing loss. *Drug Discovery Today, 10* (19), 1291-1298.

Matern, M., Vijayakumar, S., Margulies, Z., Milon, B., Song, Y., Elkon, R., … Hertzano, R. (2017). Gfi1Cre mice have early onset progressive hearing loss and induce recombination in numerous inner ear non-hair cells. *Scientific Report, 7,* 42079. doi: 10.1038/srep42079

McFadden, S. L., Ding, D., Reaume, A. G., Flood, D. G., & Salvi, R. J. (1999). Age-related cochlear hair cell loss is enhanced in mice lacking copper/zinc superoxide dismutase. *Neurobiology of Aging, 20,* 1-8.

Miller, J. D., Watson, C. S., & Covell, W. P. (1963). Deafening effects of noise on the cat. *Acta OtoLaryngologica Supplement, 176,* 1–91.

Milon, B., Mitra, S., Song, Y., Margulies, Z., Casserly, R., Drake, V., … and Hertzano, R. (2018). The impact of biological sex on the response to noise and otoprotective

therapies against acoustic injury in mice. *Biology of Sex Differences, 9*(12). doi: http://doi.org/10.1186/s13293-018-0171-0

National Institute of Health (NIH). (2017). Genetics home reference: Your guide to understanding genetic conditions. Retrieved from https://ghr.nlm.nih.gov/condition?initial=h#

National Health and Nutrition Examination Survey (NHANES), 2011-2012. (2016). Analysis reported in *JAMA Otolaryngology – Head & Neck Surgery*,

National Institute for Occupational Safety and Health (NIOSH). (1998). Criteria for a recommended standard: Occupational noise exposure. DHHS (NIOSH) Publication Number 98-126. Retrieved from https://www.cdc.gov/niosh/docs/98-126/

Ohlemiller, K. K., Jones, S. M., & Johnson, K. R. (2016). Application of mouse models to research in hearing and balance. *Journal of the Association for Research in Otolaryngology, 17,* 493-523. doi: 10.1007/s10162-016-1589-1

Ohlemiller, K. K., McFadden, S. L., Ding, D. L., Flood, D. G., Reaume, A. G., Hoffman, E. K., … & Salvi, R. J. (1999). Targeted deletion of the cytosolic Cu/Zn-Superoxide Dismutase gene (Sod1) increases susceptibility to noise-induced hearing loss. *Audiology & Neuro-Otology, 4*, 237-246.

Ohlemiller, K. K., Wright, J. S., & Heidbreder, A. F. (2000). Vulnerability to noise-induced hearing loss in ‘middle aged’ and young adult mice: A dose-response approach in CBA, C57BL, and BALB inbred strains. *Hearing Research, 149*(1-2), 239-247. doi: http://doi.org/10.1016/S0378-5955(00)00191-X

Ou, H. C., Bohne, B. A., & Harding, G. W. (2000). Noise damage in the C57BL/CBA mouse cochlea. *Hearing Research, 145(1-2),* 111-122. doi: http://doi.org/10.1016/S0378-5955(00)00081-2

Prendergast, B. J., Onishi, K. G., & Zucker, I. (2014). Female mice liberated for inclusion in neuroscience and biomedical research. *Neuroscience & Biobehavioral Reviews, 40,* 1-5. doi: http://doi.org/10.1016/j.neubiorev.2014.01.001

Sato, T. (2006). Progressive hearing loss in mice carrying a mutation in the p75 gene. *Brain Research, 1090,* 224-234.

Schwander, M. (2009). A mouse model for nonsyndromic deafness (DFNB 12) links hearing loss to defects in tip links of mechanosensory hair cells. *Proc National Academy of Science, USA, 106,* 5252-5257.

Smith, R. J. H., Shearer, A. E., Hildebrand, M. S., & Van Camp, G. (2014). Deafness and hereditary hearing loss overview. Retrieved from the NCBI Bookshelf.

Sliwinska-Kowalska, M., & Davis, A. (2012). Noise-induced hearing loss. *Noise Health, 14*(61), 274-280.

Taylor, W., Pearson, J., & Mair, A. (1965). Study of noise and hearing in jute weaving. *Journal of the Acoustical Society of America, 38,* 113-120.

Toriello, H. V., Reardon, W., & Gorlin, R. J., eds. *Hereditary Hearing Loss and Its Syndromes.* New York: Oxford University Press; 2004.

Uchida, Y., Sugiura, S., Sone, M., Ueda, H., & Nakashima, T. (2014). Progress and prospects in human genetic research into age-related hearing impairment. *BioMed Research International*. doi: http://dx.doi.org/10.1155/2014/390601

Vahava, O., Morell, R., Lynch, E. D., Weiss, S., Kagan, M. E., Ahituv, N., … & Morton, C. C. (1998). Mutation in transcription factor *POU4F3* associated with inherited progressive hearing loss in humans. *Science, 279,* 1950-1954.

Van Camp, G., & Smith, R. J. H. (2010). The Hereditary Hearing Loss Homepage. Accessed 3/10/2017.

Von Bekesy, G. (1960) Experiments in Hearing. McGraw Hill, New York.

Wallis, D., Hamblen, M., Zhou, Y., Venken, K. J., Schumacher, A., Grimes, H. L., … & Bellen, H. J. (2003). The zinc finger transcription factor Gfi1, implicated in lymphomagenesis, is required for inner ear hair cell differentiation and survival. *Development, 130*(1), 221-232.

Weiss, S., Gottfried, I., Mayrose, I., Khare, S. L., Xiang, M., Dawson, S. J, & Avraham, K. B. (2003). The DFNA15 deafness mutation affects POU4F3 protein stability, localization, and transcriptional activity. *Molecular Cell Biology, 23,* 7957-7964.

Yan, D. (2013). Mutation of the ATP-gated P2X2 receptor leads to progressive hearing loss and increased susceptibility to noise. *Proc National Academy of Science, USA, 110,* 2228-2233.

Zelaya, C. E., Lucas, J. W., Hoffman, H. J. (2015). Self-reported hearing trouble in adults aged 18 and over: United States, 2014. NCHS data brief, no 214. Hyattsville, MD: National Center for Health Statistics.

Zimmerman, D. W. (2014). Comparative power of Student *T* test and Mann-Whitney *U* test for unequal sample sizes and variances. *The Journal of Experimental Education, 55* (3), 171-174. doi: https://doi.org/10.1080/00220973.1987.10806451

CURRICULUM VITAE

NAME: Kelley Finck

PROGRAM OF STUDY: Audiology

DEGREE AND DATE TO BE CONFERRED: Doctor of Audiology; May 2019

Secondary education:
Baltimore Lutheran High School
Towson, MD
May, 2011

Collegiate Institutions Attended	**Dates**	**Degree**	**Date of Degree**
Towson University	September 2011-May 2015	Bachelor of Science	May 2015
Towson University	September 2015-May 2019	Doctor of Audiology	May 2019

Major: Audiology

Professional positions held:

- The Hearing Assessment Center: Audiology Extern
  1447 York Rd, #312
  Lutherville-Timonium, MD 21093
- Chesapeake Ear, Nose, and Throat: Audiology Student Clinician
  23 Crossroads Dr, Ste 400
  Owings Mills, MD 21117
- ENTAACare: Audiology Student Clinician
  11085 Little Patuxent Pkwy, Ste 101
  Columbia, MD 21044
- Maryland School for the Deaf: Audiology Student Clinician
  101 Clarke Place
  Frederick, MD 21705
- Kennedy Krieger Institute: Audiology Student Clinician
  801 North Broadway
  Baltimore, MD 21205