AAV-Mediated Gene Editing for the Late-Onset GJB2 p.V37I Mutation: *Potential for Restoring Hearing in Aging Mice*

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ARTIFICIAL INTELLIGENCE PREFACE:

I found Artificial Intelligence to be a very valuable tool while writing this research grant. First off, it helped me narrow down the research articles I wanted to choose by generating summaries of the main findings. These summaries helped me decide whether the papers aligned with my personal research goals and whether I should devote time to reading them in full. This helped me save a ton of time during the literature review process. After finding and fully reading all the research papers I wanted to integrate into my final, I used AI to help generate ideas for future experiments. Although I had lots of ideas in mind, I wanted to make sure that they were focused experiments, with a clear objective. AI helped me turn my ideas about the timing of gene therapy treatment into an interesting future experiment. This also ensured I addressed a knowledge gap, so I didn't repeat an experiment that had already been done.

Some of the lab procedures described in the papers were also quite complex, and I used AI to learn more about them and how they worked, so I could confidently write about them in my proposed experiment section. I haven't been able to personally complete these experiments in the Lake Forest Lab because they are expensive and complicated, so AI helped simplify the procedures in terms I would understand. For example, I was confused about how transgenic mice and AAV vectors are generated, and artificial intelligence guided me through a step-by-step process to accomplish this in the lab. In my proposal, I also used AI to help with my technical language, especially in the proposed experiment section. I wanted to make sure the structure of my sentences sounded like a standard research grant proposal, and AI helped me make better word choices in scientific terminology. Overall, I felt AI was a great resource for learning about complex lab procedures and the decision-making process researchers must go through when designing future experiments.

Hearing loss is a common condition and is linked to mutations in the GJB2 gene, which encodes the connexin-26 channel in the cochlea. Early-onset mutations have been widely studied, but late-onset mutations, such as p.V37I, are largely unexplored. While gene therapy treatments are proving useful for treating neonatal mice, this study aims to address the efficacy of gene therapy in middle-aged to geriatric patients with the p.V37I mutation. Building on the most recent GJB2 gene therapy approach (Ukaji, 2025), this study proposes using an AAV-mediated Cas9 base-editing system to target and correct the point mutation in transgenic mice across multiple age groups. This research provides crucial insight into the influence of age on gene therapy efficacy and provides hope for expanding the therapeutic window for older patients experiencing progressive hearing loss.

PHENOTYPE

My Grandpa loves to socialize - some would argue too much! He goes to the gym not for a workout, but to talk to the people around him. He will retell the same story to anyone who will listen because he's perfected it, and he knows it will get a good laugh. He lives for those mundane interactions with strangers that most people would find insignificant. When his hearing loss progressed, I could tell that he felt disconnected from his family and friends and isolated from one of the things that brought him the most joy in life. It was disheartening to watch him sit at the head of the table at Christmas dinner without talking - not because he didn't want to, but because he couldn't hear the conversation happening just 3 feet away from him. Although physically and mentally, he was healthy in his old age, his hearing loss made him feel as though he wasn't. Seeing firsthand the profound impact that hearing loss can have on a person's daily life is what drew me to study it, particularly the effects of a single-gene mutation.

The most common gene linked to age-associated hearing loss is *GJB2*, which stands for Gap Junction Beta-2 Protein. This gene encodes Connexin 26, an essential protein for cellular communication in the cochlea of the inner ear. When this Connexin 26 protein is dysfunctional, cellular signals cannot be transmitted in the cochlea. These disrupted sound signals are difficult for the brain to interpret, thus leading to partial or whole hearing loss. Additional details on the molecular function of this protein will be explained in the next section.

Genetic hearing loss is common and increasing; by 2050, it is estimated that 2.5 billion people will have some degree of hearing loss, and 700 million of those will have debilitating hearing loss (Ma, 2025). Important characteristics of *GJB2* mutations include the severity and timing of hearing loss, as well as vestibular symptoms (e.g., balance disturbances, visual disturbances, vertigo, nausea). These symptoms differ depending on the type of mutation in the gene, but are generally divided into two categories of Connexin 26 activity: partial functioning and nonfunctioning (Sakata 2023). Partial protein function leads to less severe symptoms, whereas nonfunctional proteins lead to more severe symptoms. Hearing loss severity is categorized as mild, moderate, severe, or profound. Patients with mild hearing loss can detect sounds ranging from 21 to 40 decibels, while those with moderate hearing loss perceive sounds between 41 and 70 decibels. Severe hearing loss corresponds to a range of 71 to 95 decibels, and profound deafness applies to any sound exceeding 95 decibels (Cryns, 2004).

A common mutation that leads to a partially functional protein is p.V37I, a missense mutation at position 37 that causes a valine-to-isoleucine amino acid substitution in the connexin 26 protein. 65% of patients with this mutation had congenital hearing loss, which means they were born with this mutation. The remaining 35% of patients had delayed-onset hearing loss, indicating they acquired the mutation after birth. Environmental stressors, such as loud noise, can cause non-hereditary mutations in the *GJB2* gene (Lin, 2019). The p.V37I mutation creates a partially functional protein with a broad spectrum of phenotypes. Some individuals with this mutation show no signs of hearing loss, whereas others have severe hearing loss. In most cases, though, hearing loss is strongly correlated with age, with symptoms worsening much more in later life (Lin, 2019).

A common mutation that leads to a nonfunctional protein is 35delG, a deletion of a guanine base at position 35. This creates a frameshift mutation, in which every amino acid encoded after this mutation is altered. This is because amino acids are coded in base pairs of three, and if one of these bases is deleted, then every following group of three will be altered by one pair. This mutation leads to the production of completely nonfunctional connexin 26 proteins, resulting in total hearing loss. This mutation is most common among Caucasian populations, and it is congenital in 75-80% of cases (Li, 2023). In congenital cases, affected individuals can be homozygous or heterozygous for this mutation, meaning that either only one or both copies of the gene are affected. Mutations in both copies of the gene cause significantly greater hearing loss, while a mutation in only one copy results in milder impairment. (Cryns, 2004).

Due to the wide range of phenotypes associated with *GJB2* mutations, future research is needed to determine which gene therapies and clinical treatments are appropriate for each phenotype. For instance, how does the effectiveness of *GJB2* gene therapy vary with the individual's age at the time of administration? Is there a difference in gene therapy effectiveness between individuals with early-onset hearing loss versus adult-onset hearing loss? While some studies have shown that older mice exhibit limited recovery following gene therapy, these studies used mice with early-onset *GJB2* mutations (Guo et al., 2021). What about late-onset mutations - could gene therapy still be effective in those cases?

*This author wrote this paper for Biology 352: From Genotype to Phenotype taught by Dr. Karen Kirk.

MOLECULAR FUNCTION OF CONNEXIN 26 AND MOUSE MODELS

The *GJB2* gene is located on chromosome 13q12.11 and encodes the connexin 26 protein. Connexin 26 is a member of the gap-junction protein family and is found in the epithelial cells of the cochlea in the inner ear. A gap junction acts as a direct channel between two cells and is composed of six subunits, which combine with another six-subunit protein on the other cell to form a pore between the cells (Ma, 2025). These channels differ from other ion channels because they form much larger pores that allow signaling molecules to pass through. If these subunits become dysfunctional due to mutations in their genetic code, they cannot form fully functional channels. In addition, if the channels are deformed, lysosomes in cells degrade them, even if they are only partially active (Xu, 2023). Degradation and loss of connexin 26 channels have catastrophic impacts on cellular communication and function in the inner ear.

Connexin 26 is essential for generating electrochemical gradients that facilitate potassium cycling and the transfer of signaling molecules (Ke, 2025). The scala media, one of the major fluid compartments in the cochlea, must maintain a high K+ concentration and provide a positive potential to allow passive flow of K+ into the hair cell mechanoreceptors. These hair cell mechanoreceptors become depolarized when K+ flows through them, initiating a chain of events that eventually transmits the electrical signal the brain interprets as sound. ATP also acts as an important signaling molecule in the cells of the inner ear, serving as an energy source to actively pump K+ ions into the scala media through connexin 26 channels and to regulate channel activity (Zdebik, 2009). The mechanism of hearing is complex, but the main takeaway is that cell signaling is essential for converting sound waves into signals the brain can interpret, and the connexin 26 protein is vital for this process. Mutations in the *GJB2* gene that encode this protein have been widely studied using mouse models to investigate its relationship to different severities of hearing loss and to develop potential gene therapy options.

A target ablation of the connexin26 protein in the epithelial cells of the inner ear was completed in CRISPR-generated knock-out mice to prove the overall importance of this gene function (Cohen-Salmon, 2002). After only 14 days, the supporting cells and cochlear hair cells died. Apoptosis occurred first in the supporting cells, which hold hair cells in place and maintain ion balance. Next, the inner hair cells (IHCs), which convert sound vibrations into electrical signals, underwent cell death. Shortly after, outer hair cells (OHCs), which amplify sound, underwent cell death. The death of these cells leads to altered epithelial cell shape in the cochlea and to eventual Corti collapse, a small organ in the cochlea. Despite this cell death, the mice maintained vestibular function, such as balance, but exhibited complete hearing loss. This proves that the connexin 26 channel in the inner ear is directly linked to hearing, but not vestibular function. The researchers had several predictions for why the loss of connexin-26 caused this cell death. The first is that without the connexin channels, a toxic buildup of potassium occurs around the hair and supporting cells. This overstimulates cells, leading to oxidative stress that triggers the extrinsic cell death pathway. In this pathway, external stimuli bind to death receptors on cells, triggering a cascade of events that culminates in apoptosis. When the supporting cells die first, the hair cells cannot survive (Cohen-Salmon). These results provide a strong foundation for future studies on the impact of gene-knockout timing and on how the phenotypes of specific frameshift or point mutations differ from those of an entire gene knockout.

After determining that the *GJB2* gene causes hearing loss, tamoxifen-induced knockout mouse models were generated to investigate how the timing of a *GJB2* mutation influences hearing loss. They first created a transgenic mouse with the *GJB2* gene surrounded by loxP sequences. When tamoxifen was administered, it activated the Cre recombinase enzyme, which cut the loxP sequences, thereby removing the *GJB2* gene. They found that when the *GJB2* gene was deleted before the inner ear was fully developed (at 1 day postnatal), the mice experienced more severe hearing loss than when the gene was deleted 14 days postnatal, after inner ear development (Guo, 2021). The severe structural changes in the cochlea that occurred during early gene deletion imply that the connexin 26 protein is an essential part of inner ear development. Another important finding was that hair cells, which cannot regenerate once lost, remained intact for 2 months post-*GJB2* deletion, which provides hope for future gene therapies, as gene therapy is not an option if the hair cells are already deteriorated. To begin investigating gene therapy, the researchers used an adeno-associated virus with a healthy copy of the *GJB2* gene and injected it directly into the cochlear nerve. Although connexin 26 expression increased, hair cells began expressing the protein, leading to their degeneration and worsening hearing loss (Guo, 2021). Although this research did not restore hearing, it lays a foundation for developing more precise and targeted gene therapies.

To develop precise gene therapies, it is important to understand the different types of mutations in the *GJB2* gene, particularly the optimal timing of gene therapy administration for each mutation type. Frameshift mutations lead to alterations of every amino acid coded after. The 35delG and 235delC mutations are common frameshift mutations that occur in the *GJB2* gene in humans. Knock-in mice were developed by constructing CRISPR-edited embryonic stem cells carrying these mutations, injecting the cells into blastocysts, and then implanting the blastocysts into female mice to develop. The mice with these frameshift mutations showed early-onset hearing loss that progressed into profound deafness (Li, 2023). Because this mutation leads to such early hair cell loss, it would be difficult to develop a gene therapy that could effectively restore hearing. Other types of mutations, however, lead to a much slower onset of hearing loss and offer greater potential for gene therapy to restore function.

Point mutations, which are the change of one base pair, can have less dramatic effects on the function of the connexin 26 protein. The p.V37I mutation is a missense mutation that substitutes a valine for an isoleucine at position 37 in the amino acid sequence and has been studied using knock-in mouse models. Transgenic mice carrying the mutation were generated from an embryo, and they found that this amino acid substitution led to no significant hair cell loss but did decrease the length of gap junction plaques. These gap junction plaques are clusters of Connexin 26 channels where much cellular communication occurs. As these plaques diminish, endocochlear potential decreases, leading to mild, progressive hearing loss as potassium and other signaling molecules accumulate. Because there was no loss in hair cells or the motor proteins in the inner ear, this change in endocochlear potential is the predicted result of missense mutation-induced hearing loss (Lin, 2019). Given that hair cells are not lost by this mutation, it offers hope for gene therapy interventions to restore connexin 26 function and thereby restore hearing.

Gene therapy is often delivered inside viral vectors. Adeno-associated virus (AAV) vectors are used because they do not target dividing cells, reducing the likelihood of adverse side effects. To study the efficacy of AAV-mediated gene therapy, researchers generated complete *GJB2* knockout mice and, shortly after birth, surgically injected the gene therapy vector into the cochlea. The DNA remained in the nuclei of cochlear epithelial cells, where it was transcribed into mRNA and eventually translated into functional Connexin 26 proteins. This gene therapy restored connexin 26 expression in the mice, despite not actually being integrated into the mice's genomes via a double-stranded break. (Iizuka, 2015). This study highlights the potential of gene therapy, but doesn't acknowledge that many people receiving the treatment would not receive it shortly after birth or would have the genotype for a complete knockout. In one of the most recent and groundbreaking studies regarding treating *GJB2* hearing loss caused by a point mutation, viral vectors were utilized to deliver gene therapy to edit the mutated DNA in the genome. Researchers developed a catalytically impaired Cas9 called Cas9 Nickase, which induces a single-strand break at the point mutation. A deaminase enzyme then edits the base pair to fix the mutation. This system targeted the R75W point mutation, which replaces arginine at position 75 with tryptophan. The goal was to target the single-base-pair mutation and correct it in all cochlear cells. They found that after administering the vector in vivo to mice on postnatal day 1, functional Connexin 26 was partially expressed,

indicating that the gene therapy partially reversed hearing loss (Ukaji, 2025). This exciting research opens up a whole new set of possibilities. Now that we know the inner ear can be treated with in vivo AAV-mediated gene therapy systems to edit and restore gene function in the context of point mutations, this approach can be applied to a plethora of more specific studies. For example, can the point mutation p.V37I, which is a common mutation linked with progressive adult-onset hearing loss, be treated with an AAV-mediated system at a variety of ages besides just postnatal day 1?

FUTURE EXPERIMENT

AAV-Mediated Gene Editing for the Late-Onset GJB2 p.V37I Mutation: Potential for Restoring Hearing in Aging Mice

SPECIFIC AIMS

Building on recent advancements in AAV-mediated gene editing in the inner ear (Ukaji, 2025), I plan to expand this research to investigate whether hearing loss associated with the *GJB2* gene p.V37I mutation can be prevented or restored in aging mice. The p.V37I mutation is a common cause of progressive adult-onset hearing loss, yet the applicability of gene therapy for this mutation has not been studied in older mice. I hypothesize that AAV-mediated gene editing will be effective at halting the progression of mild adult-onset hearing loss by restoring Connexin 26 expression in aging mice with the p.V37I mutation. By developing transgenic mice carrying this mutation, we can evaluate the efficacy of AAV-mediated CRISPR-based treatments across different age groups. This information will provide valuable insights into assessing the therapeutic window for treating late-onset hearing loss, particularly in determining whether older mice with established hearing loss can halt progression, restore Connexin 26, or even experience hearing recovery.

EXPERIMENTAL PROPOSAL

The type of gene mutation, the timing of gene therapy administration, and the age of patients receiving the treatment can affect its efficacy. We know that *GJB2* mutations that cause nonfunctional connexin 26 proteins are detrimental to inner ear development, resulting in severe hearing loss. In comparison, *GJB2* gene mutations that result in partially functional connexin 26 proteins are not detrimental to inner ear development and cause milder, progressive hearing loss. In cases of these less severe point mutations, there was a window for therapeutic intervention to restore hearing loss, as the inner ear's structural integrity remained intact (Guo, 2021). The p.V37I mutation would therefore be a good candidate for a future study, as mice with the mutation have normal cochlear development and hearing at birth but develop progressive hearing loss over time - very similar to how it presents in humans. It is also the most common mutation associated with adult-onset hearing loss in East Asian Populations, so this work could be applied to many people (Lin, 2019). Recent advancements in inner ear AAV-mediated gene editing demonstrate that in vivo editing of a point mutation is feasible, restoring Connexin 26 and partially rescuing hearing in young mice. This research, although exciting, has yet to show implications for older mice with late-onset mutations (Ukaji, 2025). These findings justify the immense promise of my proposed future study and make it an essential next step in hearing loss research. I will structure the gene-editing procedure very similarly to that in Ukaji's recent 2025 study, "AAV-mediated base editing restores cochlear gap junction in *GJB2* dominant-negative mutation-associated syndromic hearing loss model". The knock-in mouse model with the p.V37I mutation is the first and essential step in my experiment. A previous study by Xin Lin successfully generated a knock-in mouse with this mutation, and I will follow their procedure to ensure I also generate a successful animal model (Lin, 2019). To accomplish this, I would locate the precise base pair 109 in the mouse *GJB2* gene that corresponds to the human *GJB2* and induce a mutation in embryonic stem cells. Homologous recombination is the most common and precise method to induce these mutations. This process involves creating a DNA construct vector containing the mutation using site-directed mutagenesis. Oligonucleotide primers carrying the p.V37I mutation are designed, and PCR is used to amplify the *GJB2* gene. Because the primers include the mutation, the amplified *GJB2* gene will also contain the mutation. To ensure that only amplicons containing the mutation are inserted into embryonic stem cells, a process called restriction enzyme digestion is used to recognize and cut amplicons lacking the mutation. Gel electrophoresis can be used to extract the longer (uncut) amplicons with the mutation. Then I will use Topo-cloning to integrate the mutated gene into a plasmid, which is then taken up by E. *coli*. E. *coli* bacteria with the plasmid will grow colonies on an ampicillin plate, since the plasmid will also contain an ampicillin resistance gene. The process amplifies the mutated gene, which can then be isolated from E. coli cells and purified to high concentration. The sequence will be verified with Sanger sequencing before inserting into embryonic stem (ES) cells.

The plasmid vector will be introduced into the ES cells by electroporation. The ES cells will be placed in an electroporation buffer, to which the plasmid DNA will be added and mixed. An electroporation machine applies an electric field to the solution, temporarily making the ES cell membranes permeable and allowing the DNA plasmid to enter the cells. The ES cells are removed from the buffer mixture and incubated for at least 24-48 hours to allow DNA repair processes to incorporate the plasmid into their genome. Like Topo-cloning in E. coli cells, because the plasmid in the ES cells contains an ampicillin resistance gene, we can incubate the ES cells on ampicillin-containing plates. The surviving colonies should harbor the mutated *GJB2* gene, and these cells will undergo Sanger sequencing of *GJB2* to confirm the presence of the proper p.V37I mutation. Then, the modified ES cells will be injected into blastocysts, which are early mouse embryos. Fertilized blastocysts develop in the female mouse for about three to four days before euthanizing the mouse, dissecting out her uterus, and flushing the blastocysts onto a petri dish. These blastocysts are placed under a microscope, where a micropipette is used to inject between 10 and 20 of the edited ES cells directly into the inner cell mass of the blastocyst. The target ES cells inserted will integrate into the developing mouse fetus, creating a chimeric mouse with a mixture of normal and modified cells. The blastocysts are then implanted directly into the uterus of a surrogate mouse. In 19-21 days, the surrogate will deliver our transgenic mouse model. This mouse model can be tested for the presence of modified cells by taking blood or tissue samples and completing sequencing. These new chimeric mice can be bred together to produce offspring with the mutation, rather than going through the intensive process of creating them from scratch.

Now that the knock-in model has been generated, we must develop an adeno-associated virus (AAV)-mediated editing system to target the p.V37I mutation. Takeo Ukaji developed this system to target their *GJB2* point mutation of interest, R75W, and I plan to use a version of their editing technology for my experiment (Ukaji, 2025). In the p.V37I mutation, the base change that leads to the amino acid change is Guanine to Adenine at base position 109. Instead of using a standard Cas-9 enzyme to induce a double-stranded break, a catalytically impaired Cas9 paired with a base editing enzyme will be used to chemically change the Adenine back to a Guanine at that position. This catalytically impaired Cas9 is called Cas9 nickase, which induces a small cut in only one strand of the target DNA. Like standard Cas9, Cas9 nickase also uses a guide RNA to target a specific mutation in the *GJB2* gene. Once cut, the deaminase enzyme completes the base editing. The specific deaminase that converts A to G is called adenine deaminase. The Cas9 nickase and deaminase combination must be put into an AAV vector. This impaired Cas9 and adenine base editor is more compact than the standard CRISPR system, allowing the entire complex to fit into one vector. The smaller size of the editing system, along with an AAV vector specifically targeting cochlear supporting cells, offers greater therapeutic potential than past techniques. Once a genetic mouse model and the AAV vector have been developed, we can begin assessing the therapeutic window for treating late-onset hearing loss. Because my experiment focuses on efficacy across different age groups, I plan to test 6 age groups. Group 1 will be of neonatal mice at postnatal day 1 (P1). Group 2 will be juvenile mice at 2 months of age. Group 3 will be young adult mice at 5 months. Group 4 will be mature adult mice at 8 months. Group 5 will be middle-aged mice at 16 months, and Group 6 will be geriatric mice at 22 months.

To deliver the AAV-mediated gene therapy to cochlear cells, we will use a technique called round window membrane (RWM) injection. The round window membrane is a thin membrane at the base of the cochlea, and when treatment is injected here, it can reach cells inside the inner ear. Different surgical approaches must be used for each age group due to variations in the inner ear structure. This will ensure that the gene therapy is reaching the correct epithelial cells. For example, younger mice have delicate membranes, whereas geriatric mice have thick membranes that are harder to inject. Smaller needles should be used for the younger mice, and larger ones for the older ones. In all groups, the surgery is completed by making a small incision behind the ear and using a microscope to locate the RWM. The treatment is then injected, and the incision site is sutured closed. Therapy effects can vary, so I would like to assess auditory function in the mice pre-treatment and at weeks 2, 4, and 8 post-treatment. Auditory brainstem response (ABR) will be used to assess this function, which tests how well sound waves travel from the ear to the brainstem. We will gradually reduce the sound intensity delivered to the mice in a plastic box, and once no further waves are detected by the ABR machine, the machine will indicate the quietest sound the mouse can hear. In theory, after gene therapy is administered, hearing loss should halt or even be restored, and mice will be able to hear lower sound intensities than they would without the treatment. After the last auditory function assessment at 8 weeks, mice will be euthanized and have their cochlea removed. The connexin 26 proteins in cochlear cells will be stained with colored antibodies using immunohistochemistry to visualize the amount of connexin 26 in the cells. Brighter staining indicates greater levels of connexin 26, indicating higher expression.

CONTROLS

For each age group, there should be three control mice: two negative and one positive. Control 1 (negative) should have mutant mice untreated with the AVV vector. This control will act as the baseline for the disease phenotype. This mouse will confirm that any improvements in hearing are due to the treatment, and not random or spontaneous chance. Control 2 (negative) should be treated with an AAV vector lacking the Cas9 system. This will ensure that the vector's toxicity does not cause detrimental effects or otherwise influence the disease phenotype. Control 3 (positive) will be wild-type mice without the mutation. This mouse represents a normal healthy phenotype that shows what normal ABR and connexin levels should be in each age group. If we observe an increase in ABR and connexin 26 expression levels after gene therapy, we can conclude that high editing efficiency and hearing restoration are possible in that age group. If we observe no increase but also no decrease in ABR and connexin 26 expression level, we could conclude that further deterioration of connexin 26 was prevented, even if not reversed or fully restored. If there was a decrease in ABR and connexin 26 expression, we could conclude that the gene therapy was ineffective at preventing deterioration or restoring hearing loss. Overall, I think this research will confirm that early intervention is better and also show that late intervention prevents further deterioration of connexin 26 and cochlear hair cells.

CONSIDERATIONS

The most important consideration is the variability in mouse models due to age differences. Immune response increases with age, which could lead to the older mice having more tissue damage or higher rates of mortality due to AVV-related complications. In addition, cochlear vulnerability increases with age, and if hair cells are lost, *GJB2* gene therapy treatments would not prove useful. To prevent cochlear damage, surgical procedures must be done extremely carefully in older groups. We must also consider that older cochlear implants may take longer to respond to gene therapy due to slower healing rates. Gene therapy transduction efficiency also decreases with older age, so increasing the vector dose may help combat this issue. Sample size is important in this experiment, too, especially given its long-term nature. Ensuring that many transgenic mice are generated per group will ensure that, even if mortality rates are higher than expected within groups, there will still be enough participants to obtain statistical data. His study aims to determine whether an AAV-mediated gene-editing system can target the p.V37I mutation in the *GJB2* gene to restore connexin-26 function and prevent or even reverse hearing loss. Mild, adult-onset hearing loss is often not addressed clinically until patients are geriatric, which is why I propose using six different age groups to test the efficacy of the gene therapy, specifically focusing on whether older models respond well to treatment. Efficacy can be confirmed through auditory brainstem response tests and connexin-26 expression levels.

Studying the efficacy of gene therapy across multiple ages could provide insight into when it should be administered for optimal results. It would also be the first study done for this specific mutation, investigating whether geriatric individuals with late-onset hearing loss can experience restoration or prevention of further hearing loss with this gene therapy approach. The findings could offer hope to patients who have already missed the window for early intervention treatment. Cohen-Salmon, M., Ott, T., Michel, V., Hardelin, J. P., Perfettini, I., Eybalin, M., Wu, T., Marcus, D. C., Wangemann, P., Willecke, K., & Petit, C. (2002). Targeted ablation of connexin26 in the inner ear epithelial gap junction network causes hearing impairment and cell death. Current biology: CB, 12(13), 1106–1111. https://doi.org/10.1016/s0960-9822(02)00904-1

This study completes a full *GJB2* gene knockout and focuses particularly on the effects of cochlear cell death and its mechanisms. Oxidative stress, apoptosis, activation of caspase-activated pathways, and overall metabolic stress caused by a *GJB2* knockout result in loss of cochlear hair cells, leading to irreversible hearing loss.E., Murgia, A., Huygen, P. L., Moreno, F., del Castillo, I., Chamberlin, G. P., Azaiez, H., Prasad, S., Cucci, R. A., Leonardi, E., Snoeckx, R. L., Govaerts, P. J., Van de Heyning, P. H., Van de Heyning, C. M., Smith, R. J., & Van Camp, G. (2004). A genotype-phenotype correlation for GJB2 (connexin 26) deafness. Journal of Medical Genetics, 41(3), 147–154. https://doi.org/10.1136/jmg.2003.013896

This review examines the genotype-phenotype correlation in hearing loss caused by mutations in the *GJB2* gene. It classifies hearing loss severity by decibel measurements and considers homozygous mutations to be more severe than heterozygous mutations. Idmore, J. M., Cimerman, J., Prieskorn, D. M., Beyer, L. A., Swiderski, D. L., Dolan, D. F., Martin, D. M., & Raphael, Y. (2021). *GJB2* gene therapy and conditional deletion reveal developmental stage-dependent effects on inner ear structure and function. Molecular therapy. Methods & clinical development, 23, 319–333. https://doi.org/10.1016/j.omtm.2021.09.009

This study found that when the *GJB2* gene was deleted in mice during early stages of inner ear development, they exhibited severe hearing loss, whereas when the gene was deleted after initial inner ear development, they exhibited mild hearing loss. Losing connexin-26 function early in development leads to a loss of structural integrity in the cochlea, so when they used gene therapy with viral vectors to reintroduce a functional *GJB2* gene, they found it was most useful when administered in younger, less developed mice, with limited recovery in older mouse models. K., Gotoh, S., Sugitani, Y., Suzuki, M., Noda, T., Minowa, O., & Ikeda, K. (2015). Perinatal *GJB2* gene transfer rescues hearing in a mouse model of hereditary deafness. Human molecular genetics, 24(13), 3651–3661. https://doi.org/10.1093/hmg/ddv109

Perinatal gene transfer using viral vectors during the prenatal stage restored connexin 26 function in mice at birth or shortly before. Rather than using gene editing, a functional copy of the gene is introduced to compensate for the dysfunctional one in the mouse's DNA. Sun, Y. (2025). Regulatory mechanisms of connexin26. Neuroscience, 570, 9–15. https://doi.org/10.1016/j.neuroscience.2025.02.027

This is the most recent review of research on the regulatory mechanisms of the connexin 26 protein, both pre- and post-transcriptionally. Transcription factors, microRNAs, ubiquitination, and calcium ion concentration can all affect the protein's functionality. This paper exemplifies the importance of understanding the molecular biology aspect of disease, specifically, electrochemical gradients in the inner

ear. ao, R., Yin, X., Wang, D., Cheng, Y., Huang, B., Wang, L., Yan, M., Zhou, J., Zhao, J., Tang, W., Wang, Y., Wang, X., Lv, J., Li, J., Li, H., & Shu, Y. (2023). The pathogenesis of common Gjb2 mutations associated with human hereditary deafness in mice. Cellular and molecular life sciences: CMLS, 80(6), 148. https://doi.org/10.1007/s00018-023-04794-9

This paper examines a complete *GJB2* gene knockout, specifically the common mutation in Caucasian populations, *35delG*, which deletes a guanine base at position 35. This creates a frameshift mutation, in which every amino acid encoded after this mutation is altered. This mutation leads to a completely nonfunctional connexin 26 protein, resulting in early-onset complete hearing loss. This showcases that although there is a connexin 26 protein, it is completely dysfunctional due to this mutation.

Lin, X., Li, G., Zhang, Y., Zhao, J., Lu, J., Gao, Y., Liu, H., Li, G. L., Yang, T., Song, L., & Wu, H. (2019). Hearing consequences in Gjb2 knock-in mice: implications for human p.V37I mutation. Aging, 11(18), 7416–7441. https://doi.org/10.18632/aging.102246

This paper examines one particular mutation in the *GJB2* gene - a common missense mutation in East Asian populations found at location p.V37I that causes a valine to isoleucine substitution on the connexin 26 protein. This mutation is linked to mild, progressive hearing loss caused by loss of gap junction plaques, compared to severe, complete hearing loss in childhood that other mutations in the *GJB2* gene may cause. They are also linked to these mutations, leading to a non-congenital p.V37I mutation.

Ma, S., Chen, X., Wang, Y., & Guo, Y. (2025). Mechanisms of congenital hearing loss caused by *GJB2* gene mutations and current progress in gene therapy. Gene, 946, 149326. https://doi.org/10.1016/j.gene.2025.149326

This review discusses the actual mechanism behind *GJB2* gene mutation hearing loss, the most common types of inheritance patterns, and provides statistics to back it up. It also discusses the challenges in gene therapy and some future directions, including various delivery methods. A., Koyama, M., Urata, S., Koyama, H., & Yamasoba, T. (2023). Hearing and Hearing Loss Progression in Patients with GJB2 Gene Mutations: A Long-Term Follow-Up. International journal of molecular sciences, 24(23), 16763. https://doi.org/10.3390/ijms242316763

In this long-term follow-up study, they found that different genotypes are associated with varying severities of hearing loss. Their research helps predict how severe hearing loss will progress and aids clinical diagnosis and treatment, specifically by dividing hearing loss into two categories: partial-functioning versus non-functioning connexin 26. Tsutsumi, H., Nakagawa, R., Matsumoto, F., Ikeda, K., Nureki, O., & Kamiya, K. (2025). AAV-mediated base editing restores cochlear gap junction in GJB2 dominant-negative mutation-associated syndromic hearing loss model. JCI insight, 10(5), e185193. https://doi.org/10.1172/jci.insight.185193

This study focuses on gene therapy using adeno-associated virus-mediated base editing to correct a mutation in the GJB2 gene without inducing a double-stranded break or inserting new DNA, but rather inducing a single-stranded break and using a base editing enzyme to change a single nucleotide. This experiment showed that gene therapy can restore connexin-26 protein levels and hearing in mice treated on postnatal day 1.l, X., Xie, L., Qiu, Y., Liu, X. Z., Wang, X. H., Kong, W. J., & Sun, Y. (2023). Degradation of cochlear Connexin26 accelerates the development of age-related hearing loss. Aging cell, 22(11), e13973. https://doi.org/10.1111/acel.13973

This study emphasizes that as connexin 26 degrades in the cochlea, hearing loss progresses. The actual mechanism of connexin degradation involves lysozyme, and lysosomes were found to degrade the proteins even when they were partially functional.

Zdebik, A. A., Wangemann, P., & Jentsch, T. J. (2009). Potassium ion movement in the inner ear: insights from genetic disease and mouse models. Physiology (Bethesda, Md.),24,307–316.https://doi.org/10.1152/physiol.00018.2009

This review provides important insight into the normal functioning of the inner ear, specifically how ion movement is a necessary component of hearing. Different signaling molecules, such as K+, glucose, ATP, and Ca+2, are dependent on Connexin-26 channels, and without them, their buildup can become toxic to the cell.