Observing the Role of DSL proteins in hair cell regeneration using *C. elegans*

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The model organism, *C. elegans*, along with their OP50 were obtained from the Srinivasan Lab in Worcester MA. All testing was carried out in the lab at Mass Academy.

Abstract

Millions of individuals across the globe suffer from sensorineural hearing loss, where a low count of the hair cells in the inner ear results in this hindered hearing ability. This is due to a reduced amount of mechanoreceptors sending signals to the brain from the ear. Hair cells are microscopic mechanoreceptors that are able to transduce waves into electrical signals that are then sent to the central nervous system. Hair cells are unable to regenerate after their initial development at the fetal stage (Chen et al., 2017). The mechanoreceptors of *Caenorhabditis elegans*, or *C. elegans* , function in a similar manner and are unable to regenerate after the larvae stage. During the fetal stages of humans and larvae stages of *C. elegans*, the gene *lin-12* that codes for DSL proteins that negatively regulate the production of hair cells, is present . Two groups of *C. elegans* were used: a wildtype N2 strain and a *lin-12* gene KO. The functionality of the *C. elegans'* mechanoreceptors in each group were tested through touch assays to measure the speed of their response. *C. elegans* in the wildtype group had a lower response to the gentle and harsh touch assessments than *C. elegans* in the *lin-12* knockout group. A heightened response from *C. elegans* lacking the *lin-12* gene implies that the notch signaling pathway causes a lack of hair cell growth. Using methods found in gene therapy, such as adeno-associated viruses, removing the gene coding for DSL proteins could be a viable option for producing heightened hearing abilities in non-hearing individuals.

Keywords: *Caenorhabditis elegans*, RNA interference, *sid-1,* hair cell, sensorineural hearing loss, mechanoreceptor

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Section I: Introduction

The Mammalian Auditory System (inner ear)

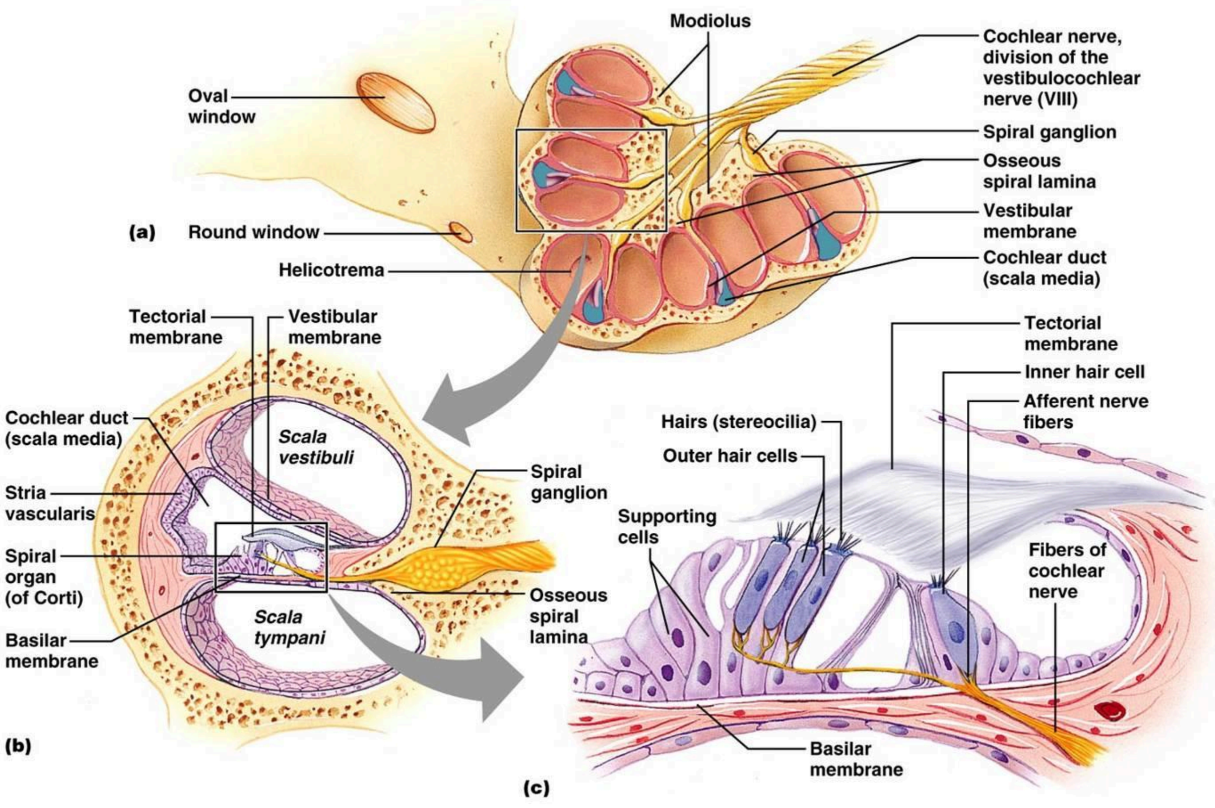

Figure 1: a) Cross-section of cochlea, b) close-up of cochlea cross section. Includes three main fluid filled sacs: scala vestibuli, scala media, and scala tympani. c) close up of scala media. Includes inner and outer hair cells as well as supporting cells. Cochlear nerve is connected to hair cells at synapses.

Inner Hair Cells and Outer Hair Cells

The hair cells (both inner and outer hair cells) both vibrate due to the movement of the basilar membrane below them. In inner hair cells, this causes stereocilia (motorsensing cilia) on their surfaces to move against the tectorial membrane, bending in the process, and consequently opening thor K+ ion channels and changing their membrane potential as the K+ ions rush in. This results in the secretion of neurotransmitters and the transduction of a signal to the central nervous system. In outer hair cells, the same process is followed, except instead of a signal immediately being sent to the central nervous system, their movement causes more vibration in the basilar membrane (a positive feedback loop), amplifying the sound and releasing otoacoustic emissions (Rim et al., 2021, 131).

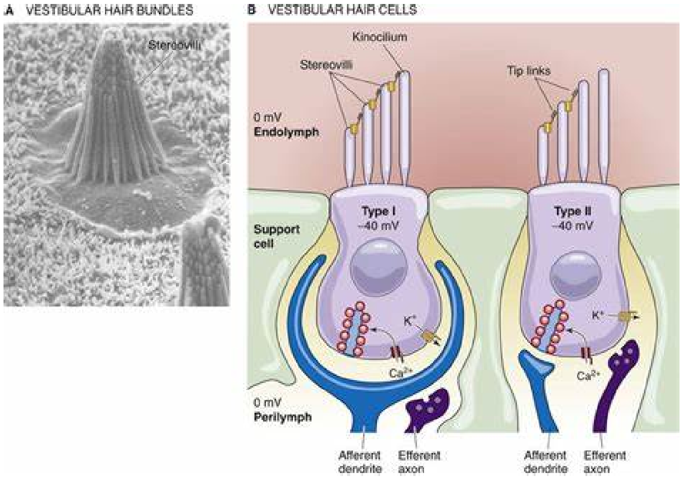

Figure 2: Inner hair cell (type 1) and outer hair cell (type 2). Calcium ions are introduced as neurotransmitters, and K+ ions are released after depolarization of the hair cell finishes. Hyperpolarization lengthens the cells (with the help of motor proteins), while depolarization shortens them.
(“Vestibular and Auditory Transduction: Hair Cells - Sensory Transduction - The Nervous System - Medical Physiology, 3rd Edition”)

Changes in the Mammalian Auditory System During Sound Input

During the transduction of a sound wave, different systems in the cochlea work together to allow the signal to be sent to the central nervous system. Some specific factors that aid outer hair cells in amplifying the imputed sound include the motor protein prestin, which aids in the outer hair cell’s electromotility by administering the depolarization of the hair cell (Dallos et al., 2006), and potassium ions that provide the hair cells with electromotility.

C. elegan Mechanosensory

C. elegans possess certain ciliated neurons with similar functions to hair cells in the human cochlea (Kramer & Moerman, n.d.). The ASH type ciliated neuron in C. elegans is stimulated by a nose touch done on the C. elegan.

C. elegan Gene Silencing

There are many different methods used to alter the genes of C. elegans. A common method is RNAi, or RNA interference, where C. elegans are simply expressed to a virus through feeding or bathed in the virus and it is able to enter and interfere with gene expression in cells (What Is RNAi - RNAi Biology | UMass Chan Medical School, n.d.). During RNAi, dsRNA, or double stranded RNA is introduced into a cell, split into two separate strands, and then the complex of the dsRNA along with the protein that split it binds to mRNA complimentary to the dsRNA strand where the mRNA is then split into bits, rendering it unusable. This means that the specific protein coded by the mRNA will no longer be created. The gene sid-1 in C. elegans is essential to the flow of dsRNA into cells (Calixto et al., 2010). Although in a biology context dsRNA is often used to administer the test subject, dsRNA is already naturally expressed within all non-neuronal cells.

The main cause of sensorineural hearing loss is a loss of hair cells in the cochlea. Overexposure to loud sounds overstimulates the cells, ultimately leading to their death. Though devices such as hearing aids or cochlear implants can help individuals suffering from hearing loss, hair cells are unable to be replenished postnatally. Consequently, individuals with sensorineural hearing loss will never be able to fully restore their original hearing or be able to hear without a special device.

Methods to regenerate hair cells involving gene therapy have been researched, though are yet to reach human testing. Currently, an adeno-associated virus, AAV-ie-K558r, has been developed to induce hair cell growth in mice (Tao et al., 2022). This virus introduces the transcription factor Atoh1 to the supporting cells in the cochlea of the mice, allowing the gene that codes for hair cells to differentiate. This transcription factor is present at the fetal stage of both humans and mice, although its presence decreases with maturity (Tao et al., 2022).. Other complexes have been known to affect hair cell expression, although additional research is needed to determine the efficacy of virus-induced expression of ATOH1.

A complex known to negatively regulate the production of hair cells, the notch signaling pathway, is a promising complex to be experimented with. Thus far, it is known to regulate cell differentiation (Lewis & Stone, 2019). The effect that the absence of this pathway has on the amount of hair cells in the ear is not yet known.

To observe the effects that the removal of the notch signaling pathway (including either the DSL proteins or the notch ligands) has on the growth of hair cells, two groups of *C. elegans*, one group with and one without the LAG-2 gene (coding for DSL proteins), will undergo tests that measure their ability to detect and react to touch stimuli based on changes in their velocity and acceleration and m/s^2. The absence of DSL proteins in the Notch signaling pathway is hypothesized to decrease the speed of the *C. elegan's* response. A heightened response implies a higher amount of hair cells, while a less aggravated response implies less hair cells.

Section II: Methodology

Role of Student vs. Mentor

Processes such as pouring NGM plates were assisted by a mentor, while the actual assessments and maintenance of the worms were done by the student. Worm strains and OP50 were obtained from the Srinivasan Lab. The duration of this project included four months of research and three months of testing.

Equipment and Materials

Nematode Growth Media

The worms were grown on NGM or Nematode Growth Media. A 0.350 L solution of NGM was made from a 1 L solution of 15.0g agar, 2.4g NaCL, 2.0g tryptone, and 2.72g KH2PO4 dissolved in water, autoclaved, then placed in a 60 degree C water bath. In the 1 L solution another 0.8mL of 1MCaCl2, 0.8mL cholesterol (5mg/mL), 0.8mL 1M MgSO4, 1mL streptomycin (100mg/mL), and 1mL nystatin (10 mg/mL) is added and allowed to sit for a couple of days before pouring in OP50 on top of each plate. Worm plates were 60 mm in diameter.

Moving Worms

Before testing, the *C. elegans* were required to be moved from their feeding plate to an empty agar plate. A bacterial loop was used to pick up worms through a microscope after being sterilized with a sterilization burner. In order to replenish the population, worms were also passed onto other NGM plates with a bacterial loop using the same method, or chunked using a spatula also sterilized with flame or 70% ethanol solution.

Techniques

Gentle and Harsh Touch Simulation

An eyebrow hair was used to touch the worms and was retrieved from willing donors (Inglis et al., 2007). The hair was stuck to the end of a toothpick with hot glue then sterilized in an 100% ethanol solution before usage. The *C. elegans* were transferred from their NGM plate to an agar plate where the assessment took place. For *C. elegan* transfer, a bacterial loop was sterilized with a flame and then used to pick up individual worms from the NGM plate and place them onto the agar plate. NGM plates were not used during testing as to not trigger other neurons (ex. Basal slowing response) (Inglis et al., 2007).

Gentle and Harsh Touch Assessments

C. elegans were chosen at random as test subjects. To ensure the same force was used for each touch, the same method was used for each test subject which goes as follows: The eyebrow hair was positioned in such a way that it poked the agar, and the pivot point created did not move. The hair was then slowly placed in a perpendicular line on the worm and lifted immediately. For the harsh touch assessment, the same method was followed using a thin glass rod in place of the eyebrow hair.

Worm Displacement and Velocity Calculations

The eyepiece diameter of the microscope was set to 11 mm during all test trials. All subsequent measurements of their distance made using the videos taken were multiplied by the ratio of 11mm over the number of pixels on the screen to reach the actual measurement of the movement in millimeters. The distance traveled by the worms was calculated by placing multiple frames of the video taken into ImageJ, which then measured the abstract line traveled by the worm. These values were then divided by the 5 seconds it took to travel the calculated distance to derive their velocity.

Specific parameters were used to determine when to initiate and terminate measurement of the worm's displacement. Two different velocities were measured: the velocity during the first 5 seconds without stimulus, then the velocity for the 5 seconds following the touch. These specific times were chosen as crucial points when the worms would be at a normal speed and their fastest speed, respectively .

To ensure efficient data was used, worms that encountered other worms while attempting to escape, worms that began escaping before being probed using the proper method (as described above), worms that were not yet full adults, and worms that were accidentally impaled were not included in the results.

To calculate the worm's acceleration during the assessments, the velocity prior to receiving stimulus was subtracted from the velocity after receiving a touch. Then their difference was divided by 5 seconds again.

Statistical Tests

This process was repeated thrice with groups of 5 worms for each strain. Next, the significance of the average changes in velocity and acceleration for the gentle and harsh touch assessments was determined using a 2 sample T-test for the difference in means, with an alpha value of 0.15.

Section III: Results

Figure 2: Table of average change in velocity and accelerations overall of both groups of worms for harsh and gentle touch assessments.

	Average change in velocity GT (mm/s)	**Average acceleration GT (mm/s^2)**	**Average change in velocity HT (mm/s)**	**Average acceleration HT (mm/s^2)**
N2	0.09	0.019	0.13	0.025

SID-1	0.17	0.034	0.13	0.026

The average change in velocity was used in the statistical significance test rather than the average velocity post- touch as different worms may have different physical limitations, causing their maximum velocity (assumed to be their velocity post-touch) to vary regardless of the other independent factors included. The average change in velocity observed for the N2 strain was 0.09 mm/s while for the *SID-1*, 0.17 mm/s.

Effect of removal of DSL proteins

A gentle and harsh touch assessment was used to determine the change in velocity and the acceleration of *C. elegans*.

C. elegans reaction to touch **(velocity)**

In the N2 strain, the average change in velocity during gentle touch was calculated to be 0.09 mm/s, while in harsh touch it was 0.13 mm/s. In the *SID-1* strain, these calculations were 0.17 mm/s and 0.13 mm/s, respectively.

C. elegans aggression post - touch (acceleration)

In the N2 strain, the average acceleration during gentle touch was calculated to be 0.0856 mm/s, while in harsh touch it was 0.02549 mm/s. In the *SID-1* strain, these calculations were 0.034 mm/s^2 and 0.026 mm/s^2, respectively.

Section IV: Discussion

Sensorineural hearing loss is caused by an absence of hair cells in the cochlea, the NOTCH family of cell receptors is known to help facilitate cell differentiation, such as hair cell differentiation in the ear. It was hypothesized that a NOTCH receptor KO strain of *C. elegans* would show results pointing towards increased mechanosensory, such as an increase in the *C. elegans* velocity post-touch (after receiving mechanical stimuli). Gentle and harsh touch assessments were used to test out this hypothesis. The difference in velocity between the control and *SID-1* strain pointed towards an increase in ciliated neuron functionality in the experimental strain, as they were able to identify the mechanical stimuli earlier than the control strain and escape faster (0.09 mm/s vs 0.17mm/s). Additionally, the average velocity of the *SID-1* strain during the harsh touch assessment was lower than that of the gentle touch assessment, and also remained almost the same as that of the control strain during the harsh touch assessment. These data points signify that the worms of the SID-1 strain were moving at their fastest speeds regardless of the touch received, providing further evidence that the removal of the lag-2 protein (or *lin-12* gene that codes the protein) allows for more mechanoreceptor generation and increases overall mechanoreception. Overall, the effect of the removal of the *lin-12* gene resulted in faster velocities during gentle touch and maximum velocities during both gentle and harsh touch assessments. This effect means that the mechanoreceptors detecting touch were able to easily generate due to the removal of the gene and resulting DSL proteins.

A T test for the difference in means was used to see the significance of the means of the velocities of the 3 different test groups for each strain for gentle and harsh touch. The statistical significance of the average velocities for each group came out to have slight significance for gentle touch with a P-value of 0.08. Though for harsh touch, the P-value exceeded 0.15.

This study follows the idea in past studies of certain proteins of the LIN family of notch receptors helping to negatively regulate gliogenesis (Zhang et al., 2020). In previous studies, adeno-associated viruses were used to introduce transcription factors into cells and induce hair cell production (Tao et al., 2022), although reducing the impact of negatively regulating proteins had previously not yet been researched prior to this study.

Future Research

Future studies can use different model organisms, such as mice, to measure the difference in actual hair cell amount once removing the *lin-12* gene. These studies would provide additional and more precise evidence of the impacts of the *lin-12* gene and the DSL proteins it codes for. Even further into the study of hair cell generation, research could be done on human subjects once deemed safe. This research would help fulfill the objective of aiding individuals with sensorineural loss.

Section V: Conclusion

Overall, the objective of determining the role of DSL proteins in hair cell regeneration was reached by using *C. elegans* as model organisms due to their similar genetics in mechanosensory. Gentle and harsh touch assessments where the velocity and acceleration of the *C. elegans* were measured showed a significant difference in velocities during gentle touch between the wild type strain of worms and the *lin-12* KO. These results point towards the removal of DSL proteins permitting more mechanoreceptor, or specifically in humans, and hair cell growth/cell growth. This study helps the overall understanding of ear health and therapeutics and can be used in future research to continue the search for a solution to sensorineural hearing loss.

Section VI: References

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Section VII: Appendices

Appendix A: Limitations and Assumptions

Limitations:

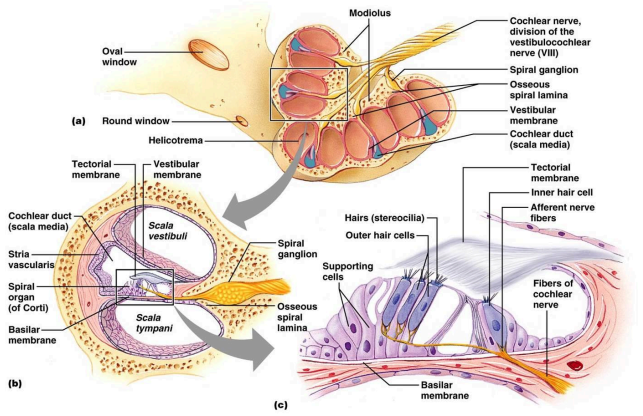
1. External funding was not available.
2. Materials were limited; strains had to be gotten from the nearby lab if they were in stock.
3. Harsh touch assessment was human-facilitated, so touches may not have always been with the same force

Assumptions:

1. The sample is representative of the population.
2. The trends observed are predictive of the future.
3. *C. elegans* moving through the plate were moving at their normal speed
4. *C. elegans* moved at their maximum speed post-touch
5. *C. elegans* already contain dsRNA for lin-12 gene mRNA (Chen et al., 2022)

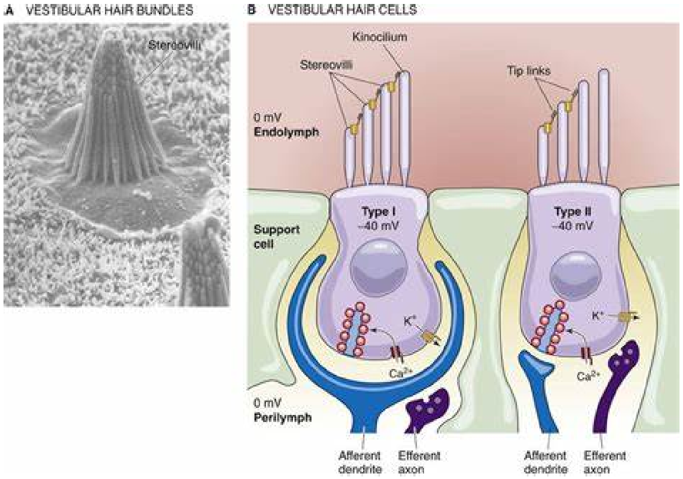
Appendix B: Figures

Figure 1:



Provides a diagram of the inner ear, from the cochlea (top), to the chambers within the cochlea (left), to the hair cells on the basilar membrane (right).

Figure 2



Provided visual of hair cells and their connection to nerve endings. This helps to understand how they act as mechanoreceptors and receive mechanical stimuli that are converted to a chemical signal.