The Effect of Cell-Cell Communication on the Polarization of Hair Cells on the Lateral Line of Zebrafish

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ABSTRACT

The most common form of deafness (sensorineural hearing loss) is the result of the inability of the human inner ear to regenerate hair cells after damage. Hair cells are responsible for transforming mechanical stimuli into neural impulses through a process known as mechanotransduction. Since zebrafish maintain their ability to regenerate these cells throughout their lives, the zebrafish lateral line is used as a model system for the investigation of hair cell development. Pairs of hair cells attain opposite polarities during development by communicating through a Notch-Delta signaling pathway soon after division from a common progenitor. To study this process, nascent hair cells were ablated while undergoing this signaling process. The study aimed to identify the duration of these signaling events as well as characterize the effect of missing a signaling partner. A posterior bias was observed in the surviving sister cells of pairs that were ablated within an hour after division occurred. This further supports the understanding of how hair bundle polarity in the neuromast is coordinated through Notch-Delta signaling. This research provides insight regarding the cells responsible for hearing impairment and has considerable implications for individuals who seek to reverse profound hearing loss.

INTRODUCTION

The most common form of hearing impairment is sensorineural hearing loss, which is characterized by the inability of mammals to regenerate hair cells (Lambert, 1994). Hair cells are vital to the process of hearing, converting mechanical sound energy into neural impulses through a process known as mechanotransduction **(**Krey, 2012). Humans lose hair cells for a variety of reasons, including exposure to loud noises or changes related to aging (Gao, 2003). The mechanosensory epithelia of the inner ear are lined with hair cells, which detect sound-evoked vibrations in the cochlea and accelerations of the head in the vestibular organs (Nadol, 1993).

Unlike humans, zebrafish maintain their ability to regenerate their hair cells indefinitely (Namdaran, 2012). Zebrafish have an organ known as the lateral line which contains six to seven neuromasts by approximately four days post fertilization (4 dpf) (Williams, 2000). Neuromasts are clusters of hair cells used to detect water movements and, consequently, the presence of approaching predators or other fish (Figure 2) (McHenry, 2009). Moreover, zebrafish are ideal organisms to study due to their small size, transparency, established mounting technique for long-term imaging, and susceptibility to anesthesia (Zhang, 2016).

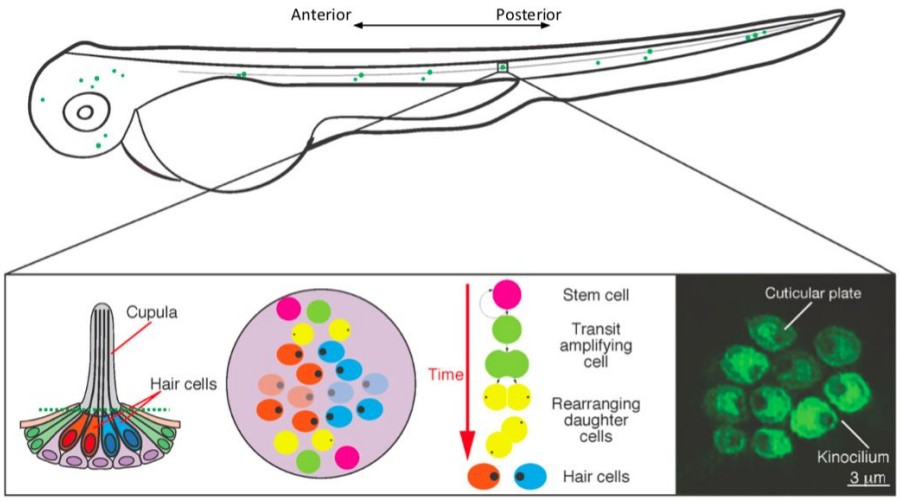

Figure 1. Neuromasts of the zebrafish lateral line.

Several clusters of hair cells are found across the body of zebrafish on the anteroposterior axis (Dow, 2018).

Each hair cell has a hair bundle, a mechanosensory organelle that contains actin-filled protrusions called stereocilia, on its apical surface (Howard, 1988). The stereocilia are organized in a staircase pattern and connected by tip-links (Figure 1) (Denk, 1995). At the taller end of the hair bundle sits the primary cilium of the cell, a microtubule-based structure termed the kinocilium **(**Li, 2003).

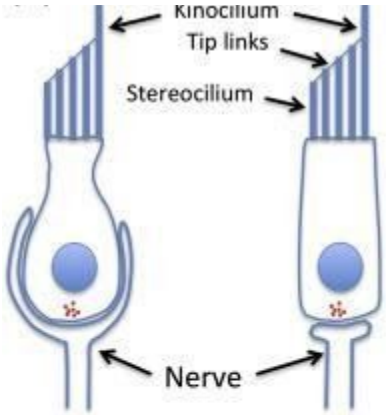

Figure 2. Hair cell diagram.

The hair bundle protruding from the apical surface of the cell consists of stereocilia connected by tiplinks and a single kinocilium differentiated by its length (Swenson,2017).

After the initial progenitor division, developing hair cells polarize either anteriorly or posteriorly (towards the head or tail, respectively) (White, 2006). The polarity of hair bundles is marked by the position of the kinocilium (Jones, 2008). The tubulin-based kinocilium can clearly be distinguished as a black dot and used to assess the polarity of hair cells because actin is fluorescently labeled to visualize hair cells (Figure 2) (Shin, 2005). In wild type fish, hair cells of anterior and posterior polarities are present in a nearly perfect 50/50 ratio **(**Fritzsch, 2003).

Hair cells are created in pairs from the division of a common progenitor (Figure 2) (Fekete, 1998). After the cell division event, the nascent pair of hair cells remain attached to each other and undergo a process of Notch-mediated lateral inhibition: each cell competes with the other to inhibit the production of Notch in their sister (Figure 3) (Ordentlich, 1998). This process creates a positive feedback loop that sets one of the cells into a high-Notch state while the other

one remains in a low-Notch state (Ma, 2008). The cell with high Notch then acquires anterior polarity while the one with low Notch acquires posterior polarity (Jacobo, 2019). Nascent hair cells remain attached for about two hours after cell division, but it is not clear for how long the lateral inhibition process lasts (Artavanis-Tsakonas, 1995). This research project aims to answer this question.

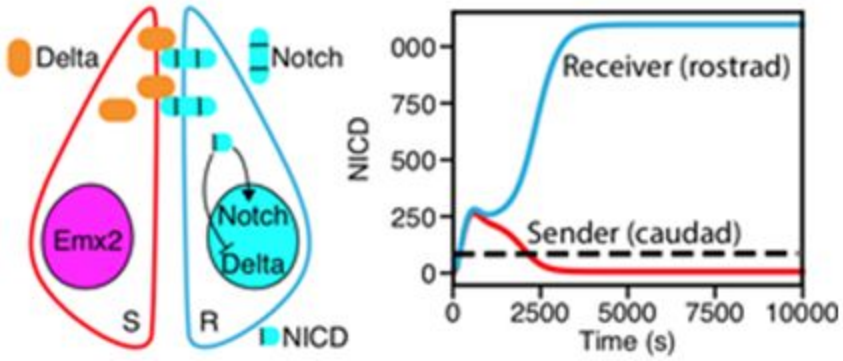

Figure 3. Notch mediated lateral inhibition.

The leftmost cell maintains a high expression of Notch causing its anterior polarity. The sister cell maintains low expression of Notch resulting in a posterior polarity (Jacobo, 2019).

OBJECTIVE AND HYPOTHESIS

An experiment was established in order to measure the duration of the signaling process between nascent hair cells. It was hypothesized that if cell communication were interrupted during the span of the lateral inhibition process, the cells would demonstrate polarity bias rather than the typical random distribution expected in wildtype fish. To perturb this process, an ultraviolet laser was used to ablate one of the sister cells in nascent pairs at variable times after division. This ablation should cause the remaining cell to lose Notch activation, adopt a low-Notch state and subsequently establish a posterior polarity.

METHODOLOGY

Preparing Zebrafish Using an Established Mounting Technique

Zebrafish ranging from 2-4 dpf were screened for *myo6b:bactin-GFP*, a transgenic line observed as a green glowing heart under a screening microscope. Only zebrafish containing Green Fluorescent Protein (GFP) were used.

GFP positive fish were left in a 1x tricaine (24 mL system water, 1 mL 25x tricaine stock) solution for several minutes until they were all completely anesthetized and immobilized. They were then mounted in a 1% agarose (10 mL system water, .01 g Low Melting Point agarose) solution and flipped upside down to be allowed to float towards the bottom of the plastic dish. Because the microscope lens images from the bottom of the dish, it was imperative the fish be as close to the coverslip at the bottom of the plate as possible. Once the agarose solidified, 1x tricaine with 20 mM vitamin C was used to fill the dish to keep the fish hydrated and asleep. In some instances, a second layer of agarose was used to ensure the first bubble of agarose would not float when exposed to the liquid tricaine.

Finding and Marking Neuromasts under an iSim Microscope

The microscope and laser were then both turned on and the MetaMorph computer software application was opened. Silicone oil was added to the 100x lens and the fish was placed on top and secured into place inside of a heating chamber to maintain a constant temperature of 28ºC. The fish was then found in brightfield view before switching the microscope to view under excitation light of 488 nm wavelength so the GFP-labeled hair cells could be found. Once a neuromast was found along the lateral line, its location was marked so the computer could maintain its position for long-term (24 hour) imaging.

Ablating Newly Divided Cells Using an Ultraviolet Laser

After the neuromasts were centered and focused, time-lapses were acquired of each of them with frames taken every 5 min. Periodically, the acquisitions would be paused and the neuromasts

would be reviewed to see if any divisions had occurred. When new pairs of hair cells were identified, a circular region of 20 pixels in diameter was marked on the nuclei of one of the nascent cells and illuminated with a high dose of ultraviolet laser to ablate it via induced DNA damage and apoptosis. For each ablation, the cell in the pair that was killed was randomized. The laser power and repetitions were initially calibrated to prevent collateral damage to non-targeted cells. The time of ablation and the position of the cell within the neuromast was noted. Time lapses were acquired overnight to observe the effects of ablation on the surviving sister cell. After acquisition the images were post-processed to reduce the time necessary to evaluate them.

Data Collection Using Multiple Python Scripts

A new computer program for the image processing platform Fiji was written in the programming language Python. The program loaded and displayed processed images from a certain date and allowed the user to manually decide if a timelapse contained any usable data. The criteria for a "usable timelapse" was established beforehand. Only datasets from neuromasts in which an ablation occurred and in which the polarity of the surviving sister could be observed were used. These datasets were then sorted into a separate directory for further analysis.

Another Python script was then written and run in Fiji to allow the user to manually enter observations. The program presented the previously selected timelapses and the user could manually input the total number of cells within the neuromasts, the presence of an apical surface, the presence of protrusions, the location of the surviving cell within the neuromast, the polarity of the surviving cell, and any additional comments needed for individual neuromasts. This input was then automatically written into a CSV file.

In order to identify the time elapsed between cell division and loss of cell-cell contact, an additional Python script was written and initiated in Fiji to take in manual input regarding the number of frames from division to ablation and the number of frames from ablation to cell death. If the ablation occurred at time point 0, "N/A" was entered for the first category. Otherwise, the number of frames was counted from the time a division (classified as a line separating a progenitor into two distinct cells) was observed and the ablation occurred (as noted from when

data was being collected). The number of frames between ablation to cell death was also counted. Cell death was classified as when the ablated sister cell no longer had contact with the surviving cell. This data was collected and written onto a new CSV file

RESULTS

Hair Cell Polarization in the Absence of the Sister Cell

Using the data annotated from the timelapse videos, a script was written in Python to make a scatterplot of cell polarity vs. time of ablation after division (Figure 4). This plot summarizes the main result of the investigation: when a cell was ablated within 50 minutes (or 100 frames) after the pair was observed to have divided, there was a clear posterior bias. For cells ablated later than 50 minutes after division, the resulting polarity was either anterior or posterior with equal probability. Two frames were acquired every minute.

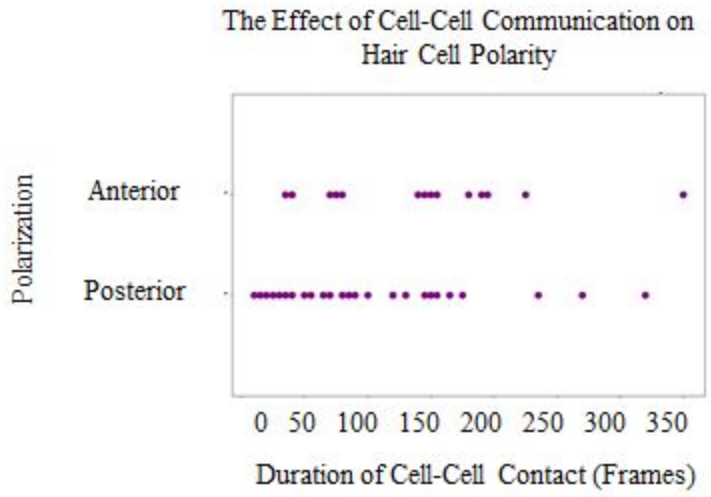

Figure 4. The Effect of Cell-Cell Contact on Hair Cell Polarity.

Each point represents the polarization the surviving cell from an ablated pair.

DISCUSSION

This study allowed the duration of the Notch-mediated lateral inhibition process that occurs between nascent pair of hair cells to be estimated. The ablation experiments show that this process is likely to happen within the first 50 min after cell division. If the contact with the sister cell is disrupted during this time window, the sister cell fails to activate Notch and becomes posteriorly polarized. Ablations occurring more than 50 min after division allow for cell-cell communication and activation of Notch to produce anteriorly polarized hair cells in about fifty percent of cases.

This finding provides important information regarding the stage at which the Notch-Delta signaling pathway operates and the extent to which the pathway controls the final polarity exhibited by the cell. This trend contradicts earlier findings published with smaller sample sizes and less methodical control of the time of ablation (Shah, 2017).

FUTURE RESEARCH

More ablations should be conducted to further validate the data. Additionally, all posterior cells in a fish and all anterior cells in a different fish might be ablated to study possible compensatory effects that might arise and modify the lateral inhibition process. Ablations at other time points, such as during the rearrangement of cells, would be interesting as well. The Notch-Delta signaling pathway requires more extensive research to better understand the mechanisms underlying cell-cell communication between hair cells and the polarization of hair cells. This basic biological research is crucial to understanding the purpose and interaction of these cells and eventually progressing towards treatment for profound hearing loss and associated conditions, such as isolation in elders.

LIMITATIONS

Due to timing restraints and the breeding capabilities of the zebrafish, only a limited sample size was utilized in this study. Additionally, shared use of the microscope limited the possible length of imaging.

Human error is also a consideration since the observed polarization of the cell was

manually input by the user.

CONCLUSIONS

Hearing loss is a serious issue in humans due to the failure of hair cells to regenerate in the inner ear. These cells are capable of regenerating in zebrafish, which are therefore ideal organisms to study hair cell regeneration. The cells polarize in opposite directions in an approximately equal ratio by lateral inhibition through the Notch signaling pathway.

An experiment in which one cell was killed to study the cells' communication was established and repeated numerous times. Early ablations led to the surviving cells exhibiting a bias towards facing posteriorly because Notch had not been activated prior to the ablation of the sister cell, leading to a lack of NICD that causes the cell to face anteriorly. Late ablations led to cells that were polarized in a relatively even ratio because Notch had already been activated in one sister and suppressed in the other sibling.

While more experiments can always be conducted to verify and better the collected results, there is no doubt that this research has great implications for the field as a whole despite the minor limitations that the research encountered.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. Hudspeth, for his continued support and constant guidance throughout the research process. Additionally, I would like to thank Dr. Jacobo and Dr. Erzberger for the time and expertise they provided as I completed my research. I would also like to thank Mrs. Frank and Mrs. Franklin, my research teachers, for believing in me and motivating me towards success. Finally, I would like to thank my friends and family for supporting me in all of my endeavors.

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