Studying Underlying Mechanisms of Usher Syndrome using Electroretinographic Recordings from Morphant Zebrafish, Danio Rerio

by
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Approved: _____
Dr. Peter O'Day

The immediate goal of this study was to gain insight into the molecular basis of Usher Syndrome. Usher Syndrome is the leading cause of hereditary deaf-blindness and its underlying mechanisms are studied at the University of Oregon using zebrafish as the model organism. The Westerfield lab investigates the disorder using cytological, molecular and behavioral analyses to unearth the molecular events that give rise to Usher Syndrome's phenotypes. This study focused on understanding the visual aspect of the disorder using the electroretinogram (ERG) as a mode of analysis. Electroretinographic recordings of morphant zebrafish—fish displaying Usher Syndrome phenotypes—were contrasted against wild-type zebrafish with hopes of gaining insight into the disorder's underlying mechanisms and possibly drawing conclusions regarding the nature of the Usher Syndrome based on experimental recordings. Analyzing the major components of the ERG waveform was a central part of this study. The a-wave, b-wave and d-wave of the ERG correspond to specific neuronal synapses within the retina—the width and amplitude of each component reveals something about the cellular activity at a particular layer within the retina. Our experimental protocol met some challenges in controlling a number of parameters, making the acquisition of meaningful data very difficult. Once a protocol with the proper combination of parameters is developed, making meaningful contributions to the study of Usher Syndrome will be possible.

Acknowledgements

I owe a tremendous thank you to Dr. Peter O'Day. He has greatly enlightened me and, more impressively, has tolerated my inexperience and inarticulate questions for months. I would also like to thank Zac Jacobs for taking me under his wing and introducing me to the experimental protocol. Thank you to Dr. Jennifer Phillips for the Morpholino injected zebrafish and to the Westerfield Lab for providing the solutions and materials used in our assays.

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Introduction

Roughly five percent of children born with hearing and vision problems suffer from a neurodegenerative disorder known as Usher Syndrome. The syndrome is eponymous after Charles Usher—a Scottish ophthalmologist of the late 19th century, who studied the causes and effects of the condition (Usher, 1914). Although Usher Syndrome affects only one in twenty-five thousand, it is the leading cause of blind-deafness in the United States (NICDC, 2008). The demographic trend of Usher Syndrome is by no means alarming; attempting to understand its underlying mechanisms, however, is a valuable endeavor to both the general scientific community and those suffering from vestibulocochlear and retinal degeneration. People enduring the most severe type of Usher Syndrome essentially live in a world devoid of illumination, color, contrast and sound. The human condition is so heavily characterized by the sensations of sight and sound, it seems unjust that anyone—by sheer genetic chance—should be unable to experience the surrounding world through these fundamental media.

Usher Syndrome is currently incurable. The primary goal of this research is to improve the understanding of the underlying mechanisms behind the neurodegenerative disorder. At present, a loss of proper gene function is thought to be the genesis of Usher Syndrome. With a deeper understanding of the mechanisms and functions of the genes giving rise to the disorder, researchers hope to develop gene therapies to restore, or improve the vision and aural capabilities of those affected by Usher Syndrome. The research backing this study, however, is focused primarily the retinal degeneration that characterizes the condition. The ensuing pages will provide necessary background

information regarding Usher Syndrome and the goal of this particular study—which is part of a much larger project—which is to contribute to the overall understanding of basic retinal physiology and function.

Three clinical types of Usher Syndrome characterize the disorder: type I, type II and type III. Type I Usher Syndrome is most severe, resulting in balance complications and deafness at birth, as well as deterioration of vision within early childhood. Individuals with type II Usher Syndrome generally have minimal problems with balance and experience moderate to profound hearing loss at birth. Deterioration of the retina is more gradual in individuals with type II Usher Syndrome, usually rendering the person blind or near blind in late childhood or early teens. Phenotypes of type III Usher Syndrome include normal hearing and balance at birth, with vision degradation delayed until young-adulthood (NICDC, 2008).

Usher Syndrome is inherited recessively, that is—its symptoms are manifested when a mutated copy of the same gene is inherited from both parents. As an autosomal recessive disorder, the mutation is not sex specific (i.e. it is found on neither the x nor the y chromosomes) and both parents must carry identical mutations on the same gene for their child to experience symptoms of the disorder.

At present, we know of nine different loci on human chromosomes that are responsible for coding Usher Syndrome (NICDC, 2008). At least two more loci have been identified as possible sources of Usher Syndrome, yet with such an array of genes associated with the disorder (and more to be discovered), research is primarily aimed at identifying all genes that lead to the syndrome. Part of the difficulty in identifying mutations that lead to Usher Syndrome is that they do not necessarily appear in an

orderly, consistent form. For example, different mutations in the same gene can result in different phenotypic responses in different people. Conversely, mutations in different genes may result in similar responses in different people.

Unless a carrier of Usher Syndrome has children with another carrier, their children are not at risk for developing the disorder. There is currently no reasonable way to test everyone for carrier status because Usher Syndrome is caused by more than one gene and work to characterize these genes is not only in progress, but also a time intensive endeavor. The inability to test large populations for their carrying status is a drawback in preventing the disorder (in potential families), making identifying the causes of Usher Syndrome extremely important. This may allow us to develop gene therapies that mitigate or repair the effects of Usher Syndrome.

As there is no cure for Usher Syndrome at the moment, early detection is the most effective recourse. Identifying the disorder in young children is important, as prompt engagement in specialized educational programs for hearing and vision loss teach the skills necessary for self-dependent living and offer support in coping with the disorder. Depending on the type and severity of the disorder, other forms of treatment include aural training, cochlear implants, instruction in sign language or Braille, as well as vocal and vestibular (balance-related) training.

My thesis centers on research into retinal phenotypes with a goal to understand the underlying mechanisms of Usher Syndrome. We plan to use molecular genetic approaches and electrophysiological assays to gain insight into the gene-protein interaction that leads to Usher Syndrome. Researchers Dr. Jennifer Phillips and Dr. Monte Westerfield at the University of Oregon mimic Usher Syndrome in zebrafish by

blocking protein translation from messenger RNA. Without the expression of key proteins, zebrafish experience physiological and anatomical irregularities that can be studied using various electrical recording tools and techniques. Using these techniques, researchers analyze and contrast physiological and anatomical irregularities against normally functioning features in healthy zebrafish—this allows researchers to expand the scope of understanding regarding the key molecular events and interactions at the core of the disorder. In the following sections, I will 1) describe the current clinical method for diagnosing Usher Syndrome in humans 2) outline retinal anatomy and physiology, as well as the significance of ERG waveforms 3) outline scientific strategies we use in the lab to investigate the causes of Usher Syndrome 4) describe our experimental approaches and methodologies 5) describe experiments and results that bear on the modes of research and 6) discuss the results in the context of the Usher Syndrome field.

Diagnosing Usher Syndrome in Humans

As individuals with Usher Syndrome suffer with bilateral severe to profound sensorineural abnormalities, it is tested for by four primary methods: visual function tests, retinal examination, hearing tests, and finally balance tests. The electroretinogram (ERG) is a safe and painless technique that can identify retinitis pigmentosa—a symptom of Usher Syndrome—by means of corneal-retinal recordings. Moreover, the ERG is the only test currently available that has the capability to recognize retinitis pigmentosa in patients before the onset of severe, to profound visual abnormalities (NEI and NICDC, 2008).

To prepare for an ERG, the patient simply wears eye patches and sits in a dark room for thirty minutes—this is done in order to dark adapt the patient and elicit highly sensitive responses to light. The researcher then tapes electrodes to the patient's forehead and removes the eye patches. After placing numbing eye drops into the patient's eyes, the researcher places specialized electrode-containing contact lenses into the eyes. The patient gazes into a spherical machine with a dark, hollow interior and a series of timed light flashes are presented. The contact lenses sense electrical signals generated by the retina as a response to the light stimulation (NEI and NICDC, 2008).

The ERG produces a graphical waveform of the change in electrical potential that reflects physiological current flow within the patient's eyes. Researchers study the principal waveforms within the ERG, which give them insight into the relative health of the patient's retina and thus enable them to make diagnoses if the waveforms deviate from the standard waveform of healthy eyes.

Early diagnosis presents some benefits: clinicians may be able to offer advice regarding the genetic aspects of the disorder, offer support in educational and vocational placement or recommend the patient to other specialists. Genetic testing for Usher Syndrome is not available primarily because all of the genes giving rise to its symptoms have not been mapped.

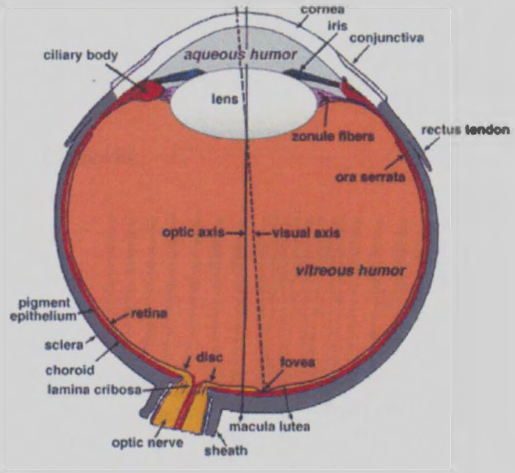
The goal of our research in the lab is to contribute to identifying and understanding how specific genes give rise to Usher Syndrome, with a long-range hope that gene therapies may be developed to treat the syndrome. To deepen our understanding of the genetic disorder, we perform analogous studies on zebrafish in the lab. Zebrafish are used as model organisms for research into genetics and vertebrate

development because of their transparent, easily accessible embryos, simple breeding and short maturation time. These are traits that facilitate experimental observation and manipulation of the organism (Westerfield, 2000). Furthermore, the zebrafish genome shows remarkable similarities to the human genome, demonstrating the relevance of zebrafish research and its implications for human health issues.

Background

Anatomy and Physiology of the Eye

The vertebrate eye consists of three layers: an outer fibrous layer, a middle vascular layer (i.e. containing blood vessels) and an inner layer. The outer layer is composed of the sclera and cornea—made of collagen fibers—that protect and give shape to the eye. The middle layer is made of the iris, the ciliary body and the choroid. The iris is the pigmented circular muscle responsible for contraction and dilation of the pupil. Separated by the iris are the anterior and posterior chambers of the eye, which produce and drain a nutrient, oxygenated solution vital to the eye's avascular regions called the aqueous humor (Dowling, 1987). The vast majority of the eye is full of a substance called vitreous humor, which also serves as a nutrient to parts of the eye that do not contain blood vessels. The choroid and ciliary body make up the vascular layer of the eyeball contain blood vessels and capillaries, which supply the eye's muscles with oxygen.



Cross sectional diagram of a vertebrate eye.
http://webvision.med.utah.edu/imagesww/draweye.jpeg

The Retina

Posterior to the outer and middle layers of the eye lies the retina. This is where "vision" begins and where abnormalities in neuronal signaling that lead to blindness most likely occur. The body of research on Usher Syndrome suggests that the retina is the source of malfunction underlying the disorder.

The vertebrate retina is a complex sensor of visual stimuli such as movement, color, contrast and varying degrees of illumination. It is comprised of three layers of nerve cells sectioned off by two layers of synaptic junctions. These cells are located at the back of the retina. From anterior to posterior, the neuronal layers are called the outer and inner nuclear layers and the ganglion cell layer—divisions between these cellular bands are made by the outer and inner plexiform layers (Dowling, 1987). Within the ganglion and outer and inner nuclear layers lie the six principal classes of neurons: receptor cells (rods and cones), horizontal cells, bipolar cells, amacrine cells, and ganglion cells. Information flows vertically from photoreceptors, to bipolar cells, to ganglion cells. This same electrical signal can also travel laterally and medially through horizontal and

amacrine cells, giving rise to a number of intricate neuronal pathways. The cells transfer electrical messages to one another in a complex sequence of events that is not yet fully understood.

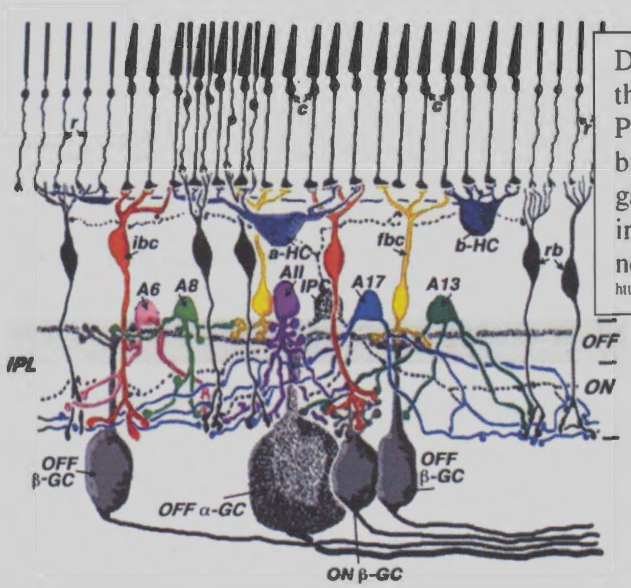


Diagram of three main layers of the vertebrate retina: Photoreceptors (outer layer), bipolar (middle layer), and ganglion cells (inner layer)send information radially to the optic nerve.
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The photoreceptor cells are responsible for light absorption and are located at the very back of the retina (Kaneko et al., 1999). Therefore, light must travel through the clear vitreous humor — which makes up the major volume of the eyeball—to excite the receptors. At the back of the retina, a synaptic crossroads between the eye's pigment epithelial layer and the pigmented photoreceptor membranes forms to enable transfer of *retinal*, a derivative of vitamin A. In photoreceptors, there are four major components that make light absorption possible: 1) an outer segment consisting of stacks of membrane bilayers containing rhodopsin, the pigment molecule that first absorbs light in the visual pathway, 2) an inner segment in which mitochondria (specialized cellular "power plants") and ribosomes (RNA-protein complexes) are found, 3) a cell body, which contains the cell nucleus, and 4) a synaptic terminal at which the visual

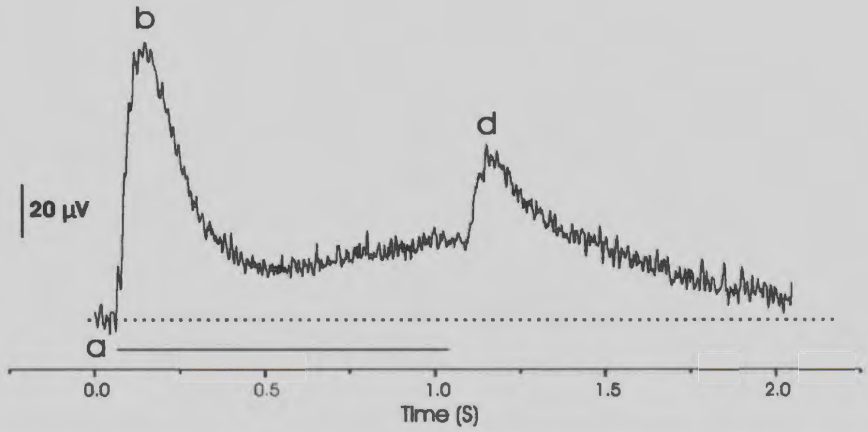
information is passed to higher order cells in the neural pathway, via neurotransmission (Dowling, 1987). The process involves conversion from an electrical to a chemical signal (transmitter release) from the presynaptic cell and generation of an electrical signal in the postsynaptic cell initiated by the chemical transmitter.

When performing an ERG on zebrafish, the focus is on three types of cells: ON bipolar cells, OFF bipolar cells and Muller glial cells. As will be explained in the next section, ON bipolar cells and Muller cells are in a depolarized state in the dark, due to the continual release of the neurotransmitter glutamate. The release of this amino acid inhibits ON bipolar cell and Muller glial cell activity and activates OFF bipolar cells. In short, ON bipolar cells and Muller glial cells are active under light conditions and OFF bipolar cells are active under dark conditions (Kolb, et al., 2003).

Muller cells—a subtype of neuronal support cells known as glial cells—are important for three major reasons. Firstly, they form myelin, a lipid protein complex that electrically insulates nerves. This action thereby enhances and regulates the speed at which electrical signals are transmitted. Lastly, they establish neuronal connections that regulate the environment's surrounding neurons, forming the "white matter" of the brain, for example ("Outer Plexiform Layer"). Not much is known about the direct role of Muller cells within the retina other than they provide retinal structure and promote synapse formation.

Significance of ERG Waveforms

When photons (packets of light) are absorbed in the retina's photoreceptors (rods and cones) a voltage change can be measured in the eye, using the ERG technique. This involves placing an electrode in the eye (with the reference electrode outside the eye in the bathing saline solution), presenting a light flash, and recording the voltage generated in the retina, using an oscilloscope and computer-aided data acquisition hardware and software. This technique reveals three principal ERG wave components: the *a*-, *b*- and *d*-waves. The ERG arises from the opening and closing of ion channels in the photoreceptors; the resulting ionic currents cause changes in the cell's electrical membrane potential, which travels to the synapse, sending information electrically and chemically to the higher order cells. These cells are many and they respond with their own changes in ionic current and electrical voltage.



Sample ERG waveform, showing a, b, and d waves. Recorded from zebrafish eye in whole animal preparation. The horizontal bar represents the 1 sec duration of the light flash. Recording was made by Phil Han in the O'Day lab.

The above image shows the three principal components of the ERG. The *a*-wave shows a negative potential, indicating light's conversion into electrochemical energy within the photoreceptors. The *b*-wave shows a positive electrical potential, which is observed directly after the *a*-wave. The *b*-wave response is due to activity of the ON bipolar cells, as well as a minor component of the Muller glial cells. Finally, the *d*-wave is a late, positive response to the offset of the light stimulus. The *d*-wave response is attributed to the activity of OFF bipolar cells (Perlman, 2001). The essential point behind reading ERG waves is that they correspond to activity in particular cell layers within the retina. Spotting differences between Morpholino and wild-type fish ERGs will provide us with more insight into the nature of neuronal interactions.

Visual Physiology

The outer segment of a photoreceptor is composed of stacks of bilipid membranes, which contain light-sensitive pigment molecules. The visual pigment is composed of a protein called opsin, which is attached to a light-absorbing derivative of vitamin A called retinal. Opsin contains seven transmembrane helices, the seventh of which binds retinal in a central pocket formed by the helices (Kolb, 2007). When light is absorbed in the outer segment of a photoreceptor, the photon energy excites electrons forming the bonds between the atoms of retinal. This addition of energy induces a change in the three-dimensional shape (isomerization) in retinal, which leads to a conformational change in opsin and a resulting change in the chemical binding properties of the pigment. Opsin is converted into an unstable molecule called metarhodopsin II, which breaks open and releases the retinal. When this break occurs, part of the metarhodopsin membrane is exposed for binding to a three subunit G-protein. When initially bound to the metarhodopsin, the G-protein is composed of alpha, beta and gamma subunits. The alpha subunit carries a bound GDP molecule (a form of energy) that is exchanged for a GTP molecule (Kolb, 2007).

When the exchange of GDP for GTP takes place, the alpha subunit is released from the beta and gamma subunits and free to travel along the inner membrane of the lipid bilayer until it makes contact with and activates another membrane bound protein called phosphodiesterase. In its active form, phosphodiesterase cleaves bonds forming a molecule called cyclic guanosine monophosphate (cGMP), which acts as a gate-opener for the sodium ion channels within photoreceptors. Hydrolysis (degradation) of cGMP

results in a closure of these ion channels and a consequent change in the retina's electrical potential, which travels along the photoreceptor membrane to the synapse.

In the dark, there is a continuous inward flow of sodium regulated by cGMP-gated sodium channels as well as an outward flow of potassium through non-gated ionic channels. The influx and efflux of these atoms in the dark causes a change in the cell's internal and external electrical voltages. In the dark, the influx of sodium ions is generally more rapid than the efflux of potassium ions, resulting in a decrease in membrane potential, which is termed depolarization. Among other things, depolarization causes voltage-gated calcium channels to open (Hille, 2001). High concentrations of calcium within the photoreceptor causes the release of neurotransmitter-containing vesicles which bind to the cell membrane and release the neurotransmitter into the synaptic cleft—the space between the terminal end of a photoreceptor and the beginning of another cell (Kolb, et al., 2003). Ultimately, the electrical response of the photoreceptor membrane travels to the synapse, triggering entry of calcium—the signal for release of the neurotransmitter glutamate, which signals the next order neuron.

Light striking the eye travels mainly through the clear, vitreous humor and is absorbed by the pigment-containing epithelial layer and photoreceptors of the retina. By virtue of their pigmented membrane, the photoreceptors are the sole class of neurons within the retina capable of light absorption (Kolb, et al., 2003). Light stimulation evokes a sequence of events in which an electrical message is sent from the photoreceptor cell bodies, through the two layers of synaptic connections, to a nerve fiber layer, which coalesces with the optic nerve, transmitting the signal to the brain for additional image processing.

Gene Function and Protein Expression

To contextualize the genetic manipulation researchers perform on zebrafish, a brief review of the form and function of protein expression is necessary. Deoxyribonucleic acid (DNA) is composed of four subunits called nucleotides, or bases. These nucleotides each have a specific complementary nucleotide they form stable bonds with in order to form the lengthy double helix. The arrangement of the bases contains the genetic instructions that guide the physiological and anatomical development of all life forms on earth. With DNA serving as a "blueprint," an enzyme called RNA polymerase transcribes the genetic code into messenger ribonucleic acid (mRNA). Other molecular machinery within the cell translates the genetic code within the mRNA into the language of proteins. The products of translation disseminate to form different structures and perform various functions throughout the cell. The steps comprising the processes of transcription and translation are highly regulated by specialized proteins. Such proteins can, for example, down- or up –regulate DNA's binding affinity for RNA polymerase, ultimately enhancing or silencing the expression of certain proteins. In physiologically normal organisms, mRNA is translated into proteins, which are then sent to specific cell sites to perform their physiological roles. Organisms that display anatomical and physiological abnormalities lack the ability to produce specific proteins. The use of sophisticated technology allows investigators to manipulate the synthesis of specific proteins—thus mimicking the protein deficiencies characteristic of Usher Syndrome—while the genetic material itself remains unchanged.

Researchers control zebrafish protein synthesis through use of Morpholino injection technology. Morpholinos are synthetic molecules composed of the same nucleotides as the genetic material—they differ only in that their phosphate-sugar backbones are modified in order to increase stability and resist breakdown. The Morpholinos are injected into zebrafish that are in the one to two cell stage of development to ensure that every cell in the mature zebrafish will incorporate the Morpholino molecules into translation. Morpholinos function by binding to complementary sections on single strands of mRNA, preventing the molecular machinery involved in translation from decoding the message found in the particular region of mRNA. Without the translation of these specific regions of mRNA, instructions for the synthesis of key proteins cannot be carried out, resulting in abnormal physiological and anatomical effects. The primary disadvantage in using Morpholino injection rather than genetically modifying the zebrafish is that the molecules become less robust over time (Gene Tools, 2009). As the zebrafish matures, the Morpholinos's inhibitory function is weakened. In any case, Morpholinos are used because of their effectiveness and because the alternative—genetic manipulation—is a difficult and expensive task.

In zebrafish, the anatomical and physiological irregularities manifest themselves as abnormal swim bladders, and faulty auditory and vestibular systems. These abnormalities dramatically affect the fish's senses of balance and motion and prevent them from swimming normally. It is therefore extremely important that these Morpholino injected fish be cared for properly, as ERG testing on unhealthy zebrafish compromises the results of the recordings.

In targeting the loci responsible for Usher Syndrome with Morpholinos, researchers can gain understanding of the anatomical and physiological effects specific proteins have on a network of cells. Investigators deconstruct a complex set of molecular interactions by inhibiting the function of one gene and the proteins it encodes and taking note of the physiological effects of this inhibition. These effects are contrasted against physiologically normal organisms in order to piece together important interactions within a cell's physiological processes. This process of comparing and contrasting the function of genes in Morpholino injected fish and uninjected fish is repeated and accordingly refined in order to gain a detailed understanding of the underlying mechanisms of Usher Syndrome.

This study specifically compared ERGs from zebrafish containing a Morpholino designed to "knock down" the expression of USH1C-encoded proteins against uninjected fish. The protein this gene encodes for is known as Harmonin, which serves as a scaffolding protein. Scaffolding proteins act as "middle men" in cell synapses, orchestrating protein-protein interactions. Harmonin has three isoforms*, each containing two or three domains that bind other proteins (PDZ domains) (Siemens, 2002). In harmonin, these PDZ domains aid in the binding of transmembrane proteins to cell cytoskeletons. In retinal photoreceptors and in hair cell synapses, Harmonin is thought to play an important role as a principal scaffolding protein.

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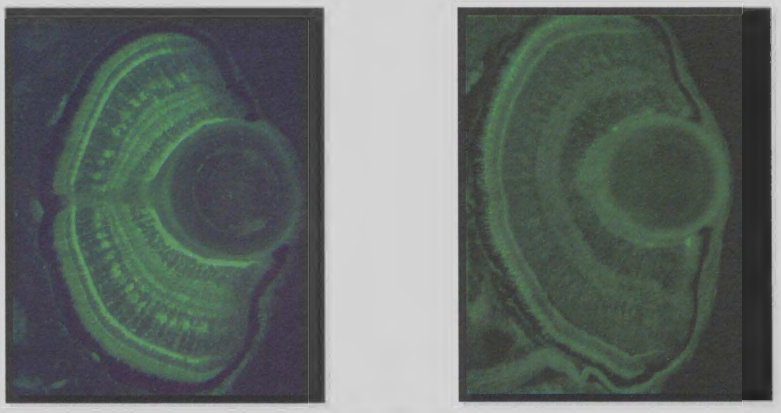
* An isoform is a protein that has the same essential function as another protein, but is encoded by a different gene. Isoforms can be thought of as "versions" of a protein.

Cytological Studies of Harmonin

At the University of Oregon, Dr. Jennifer Phillips, a postdoctoral fellow at the Westerfield lab, demonstrated that harmonin deficiencies result in abnormal swimming, balance, and visual function in zebrafish, suggesting that knockdown of the USH1C gene causes behavioral changes.



Fluorescent antibody staining of morpholino-injected zebrafish eye (right panel) and uninjected control eye (left panel) visualized in fluorescence microscope (Dr Jennifer Phillips, University of Oregon). Morpholino was generated to inhibit expression of ush1C gene, which codes for harmonin, and the antibody was directed

In addition, the USH1C morpholino injection causes clear morphological differences in the eye, as illustrated in the above images, generously provided by courtesy of Dr. Phillips. This figure shows larval eyes of five-day-old zebrafish, stained with a harmonin antibody. The image on the left corresponds to an uninjected control, while the image on the right shows the eye of a Morpholino injected fish. The figure on the right displays highly organized synapses, while the image on the left shows less defined pre and post-synaptic junctions. In the image on the left, the harmonin antibody expression is clearly localized in Muller glial cells. Muller glial cells are a subtype of neuronal support cells (Dowling, 1987), which form myelin, a lipid membrane protein complex that electrically insulates nerves, enhancing and regulating the speed at which electrical

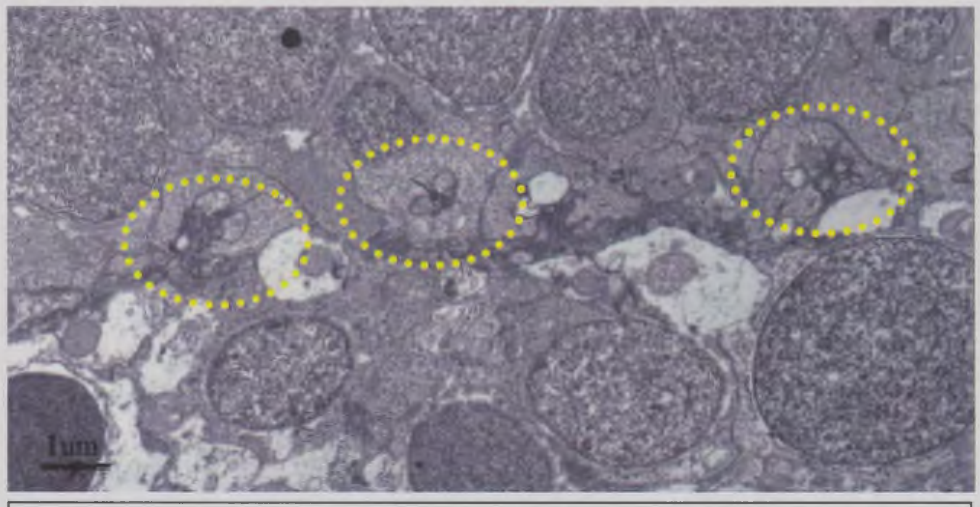
signals are transmitted. They also establish neuronal connections that regulate the environment's surrounding neurons (Kolb et al., 2003). Not much is known about the direct role of Muller cells within the retina other than they provide retinal structure and promote synapse formation. The figures show that harmonin plays a major role with respect to role in the proper development of Muller glial cells.

Dr. Phillips's finding that harmonin is expressed primarily in glial cells was unexpected because we had some preliminary indication that the electrical activity was profoundly affected by Morpholino treatment, and yet the glial cells provide only a minor component of the ERG waveform. It is therefore of great importance to characterize and establish the effect of on the ERG waveform, under a variety of experimental physiological conditions of Morpholino induced knockdown of the ush1c gene product. Since Morpholino injection results in a dramatic reduction of harmonin—which we have now observed to be highly concentrated in Muller cells—it is fair to hypothesize that the effects of this knockdown will somehow influence the ERG's *b*-wave (most likely decreasing *b*-wave amplitude). We would predict that the *a*- and *d*-wave components of the waveform would remain unaffected because these constituents of the ERG are apparently not influenced by Muller cells.

The following figure, also provided by Dr. Phillips, is an image of cone pedicles in the retina, taken by an electron microscope. Cone pedicles are the terminal ends of cone photoreceptors. Much like rods, cones transfer light induced messages to higher order neurons by releasing glutamate (Dowling, 1987). The specialized structures

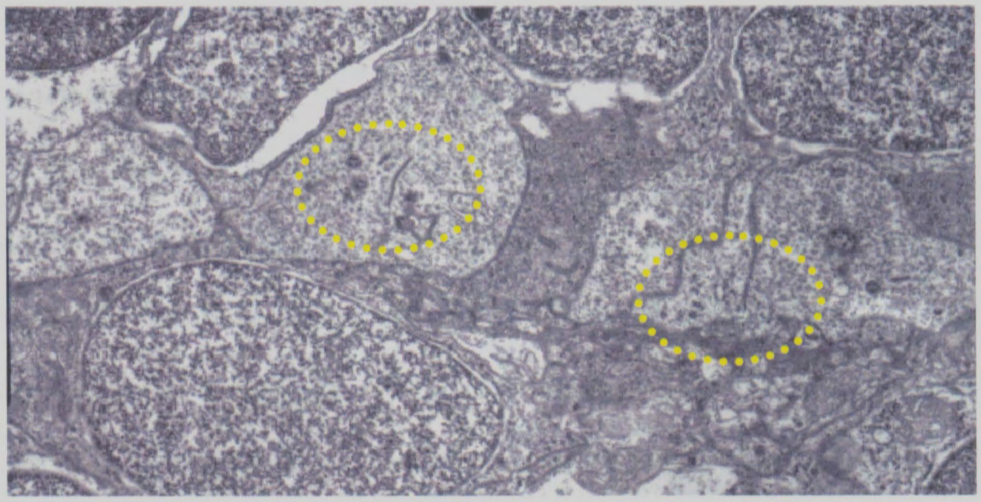
surrounding ribbon synapses, known as triads*, which are the specific sites responsible for the release of the neurotransmitter (Dowling, 1987).

The first image shows a typical triad in an uninjected control larvae. The triad shows correct symmetry, with the ribbon forming a midline between the curved postsynaptic processes. The following image shows cone pedicles of a Morpholino injected fish. Although ribbon synapses do form in morphant zebrafish, they are highly unorganized and lack the symmetry of synapses observed in the uninjected controls, highlighting harmonin's importance in another aspect of the retina.



Electron microscopic image of "triads" – synaptic regions at which photoreceptors transmit information to bipolar cells. Image is from the retina of a normal, uninjected fish. Data of Dr Jennifer Phillips (Westerfield lab, University of Oregon)

* Ribbon synapses consist of an electron-rich ribbon in the presynaptic membrane. The ribbon is generally located adjacent to an evaginated ridge of the terminal membrane and is anchored by a dense, curved band known as the acriform density. The acriform density and postsynaptic processes (bipolar and horizontal) compose the triad.



Electron microscopic image of a retina from a ush1C-morpholino-injected fish. Note that ribbon synapse regions are identifiable, but disrupted, and no triads are visible. This was observed throughout the retinal slices in ush1C-morpholino-injected fish. Data of Dr Jennifer Phillips (Westerfield lab, University of

These cytological studies demonstrate the important role harmonin plays in normal retinal anatomy and physiology. More specifically, the imaging and staining techniques described illustrate that harmonin is an essential scaffolding protein, crucial to proper synaptic function.

Materials and Methods

The following recipe was used for stock solutions:

E2 medium (15mM NaCl, 0.5 mM KCl, 1 mM MgSO4, 0.150 mM KH2PO4, 0.050 mM Na2HPO4, 0.7 mM NaHCO3, pH7.1-7.4).

MESAB (buffered 3-aminobenzoic acid methyl ester).

Animals

5 day old zebrafish raised under normal light cycle conditions (14 hours of light, 10 hours under dark conditions.

Stimulation

A one channel LED (light-emitting diode) system was used to stimulate the zebrafish retinas. A white LED light was placed above the zebrafish eye, powered by an in house 5-V driver. An electroretinography setup consisting of an Axoclamp CV 201A headstage, Tektronix oscilloscope and Clampex 8 program was used to record retinal responses. Silver-silver chloride wire within micropipettes were used as electrodes (the tips were roughly diameter of approximately 20 μm). The electrical signal was amplified and sent to the oscilloscope for graphical representation.

Procedure

We use five-day-post-fertilization (dpf) larval zebrafish for our experiments. These fish are less than the size of a human eyelash, meaning gentle handling of the fish is crucial in maintaining a viable experimental organism. The fish are dark adapted for at least 30 minutes, placed on a damp sponge and anesthetized with a muscle relaxant (MESAB). The sponge holding the fish is placed into a 35 millimeter Petri dish and positioned beneath a microscope. A glass microelectrode is then carefully placed against the fish's cornea. An LED is used as the source of light stimulus, transmitting a bright, localized flash onto the fish eye. A silver chloride wire is pushed through a glass pipette to create an electrode. The electrode is placed on the corneal surface and pushed through the vitreous chamber to be in the vicinity of the retina. Ionic chambers within the retina produce charged molecules that generate an electric field toward the electrode. When light is flashed onto the eye, the retinal cell layers and their synapses generate an

electrical response. Recall that the opening and closing of ion channels in the retina regulate the amount of electrochemical energy within the cell. The amount of electrochemical energy within retinal cells is contingent upon the intensity and duration of light stimulus. As the retina's membrane potential is subject to a wide range of light stimulus, we have developed an experimental setup that virtually eliminates the variability of light intensity. Light intensity is controlled by adding a series of glass filters in front of the light source to obtain consistent transmittance atop the eye. Variables such as light wavelength and frequency were disregarded to maintain a relative degree of simplicity.

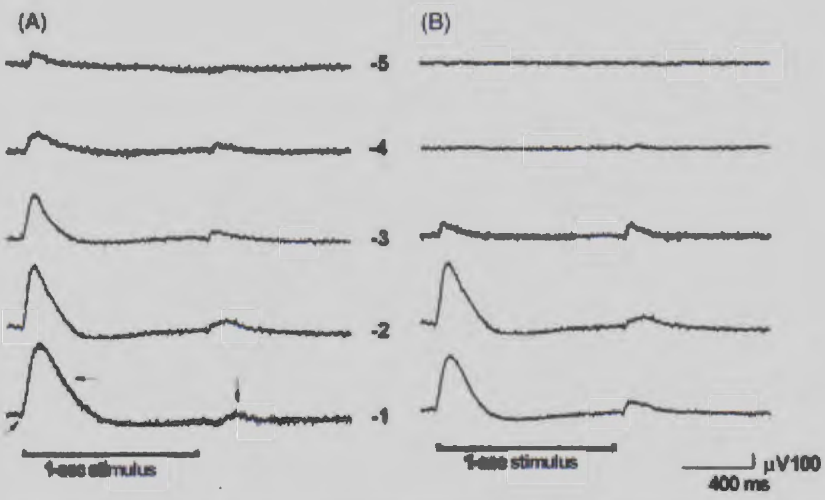
The retina's response to the light flash is carried from detected the electrode and displayed on to an oscilloscope and finally a computer, which converts the electrical message into a series of graphical waves. Studying the trends of these waves enables us to analyze electrical activity and cellular signaling within the retina.

Our procedures were built upon the work of several preceding investigations (Connaughton, 2003; Makhankov et al., 2004; Saszik et al., 1999; Wong et al., 2004, 2005).

Response vs. Stimulus Intensity

The following figure, from Makhankov et. al (2004), provides evidence that the duration and intensity of light stimulus, as well as the environment of stimulus are important parameters that can be controlled in order to obtain meaningful results. A brighter and longer stimulus will produce waves of larger amplitude and width. On the

left are responses from dark adapted fish, while responses of light adapted fish correspond to responses on the right. These figures show why we choose to dark adapt the fish—responses of dark adapted fish are simply more pronounced.



Electroretinograms recorded from whole zebrafish illustrating response-intensity relationships in dark adapted (A) and light-adapted (B) conditions. Relative intensities (as $\log_{10}$ units) are displayed between the panels. Data are from Makhankov et al (2004).

The dark adapted fish display much lower thresholds to response. Note that the *a*-wave—the initial decrease in membrane potential—is not present in the light adapted fish until light intensity has increased by a factor of 100 (-2). In short, responses can be elicited from dark adapted fish in column (A) at much lower absolute stimulus intensity because the neuronal cells are unbleached by the light (i.e. are more sensitive to stimulus because of greater chemical and electrical potentials). Although our experimental setup is aimed at recreating consistent responses such as the final waveform in column (A), I have yet to produce any.

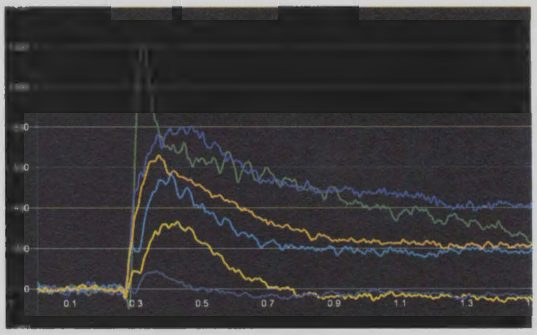
One of the reasons for this is that experiments with ush1c morphants have been postponed until the results of experiments by postdoctoral fellow Dr. Alexandra Tallafuss

and others involving these fish are undergoing publication. The other reason behind this is that the experiments run by my lab partner (Zac Jacobs) and myself have exhibited a large degree of variability. We are working with Dr. Peter O'Day to improve the experimental protocol in order to produce both consistent and meaningful results. A number of parameters such as electrode diameter, orientation of the electrode within the eye (both depth and angle of penetration), fish preparation and adaptation and positioning of the light stimulus make the acquisition of meaningful results a difficult endeavor. As such, we are working to establish a protocol with the proper combination of parameters required to generate consistent ERG responses.

Results

The figures on the next page are responses recorded by Zac Jacobs. The left-hand graph shows a typical response of a wild-type zebrafish, with the *b*-wave's maximum amplitude at roughly 300 microvolts. To reiterate the basic waveform, the initial negative response is called the *a*-wave and the large positive component is known as the *b*-wave. Within

individual fish, at a constant light intensity we have viewed responses ranging from 200 microvolts to 1200 microvolts, as shown on the right-hand graph. With so much variability in responses—especially within an individual fish—it is not possible to draw any meaningful conclusions from the ERG recordings. Despite the difficulties in obtaining significant results, past research has demonstrated that producing consistent results is possible. Improving and perfecting our experimental protocol, therefore, will be essential in making any meaningful contributions to the study of Usher Syndrome.



Electroretinograms recorded from whole zebrafish illustrating the large degree of variability in response size and shape. Relative intensity was -3 $\log_{10}$ units. Recordings were made by Zac Jacobs in the O'Day and Westerfield labs (University of Oregon) and reported in Aug 2008 in the SPUR undergraduate research symposium.

The present study is not fully developed in its experimental rigor. Much time was spent in the lab working to improve our experimental assays in order to produce and reproduce consistent results. Once the correct combination of parameters is established, our ability to contribute significant findings to the Usher Syndrome field will be possible.

Future investigations into Usher Syndrome

Understanding of ush 1c is just one part of an intricate story. The table below shows analogous human and zebrafish Usher Syndrome genes and the proteins for which

they encode. These genes serve numerous functions such as cell adhesion, transmembrane linkage, synaptic shaping and motor operation. The proper function of these proteins on the molecular level has tremendous implications on the macroscopic level. Gene products are crucial to the maintenance of sensory cells in the inner ear that transmit sound and motion signals to the brain. These loci prove to be important to the proper anatomical and physiological processes of the retina, giving photoreceptors the ability to sense light and transmit an electrochemical message to neighboring neuronal cells. The precise role some of these genes play with respect to hearing and vision in presently unknown. Research thus far has shown that mutations responsible for Usher Syndrome cause a loss of sensory cells of the inner ear and degeneration of retinal photoreceptors. Simply contrasting the harmonin expression of uninjected controls to morphant zebrafish described in the cytological study allows one to recognize the crippling effects Usher Syndrome can have on the retinal synapses.

Comparison of genes having apparent involvement in Usher Syndrome in humans with analogous genes in zebrafish and corresponding zebrafish mutants.

Usher type	Human gene	Zebrafish gene	Zebrafish mutant	Protein
USH1B	*MYO7A*	*myo7a*	*mariner*	Myosin VIIA
USH1C	**USH1C**	**ush1c**		**Harmonin**
USH1D	*CDH23*	*cdh23*	*circler*	Cadherin precursor
USH1E				unknown
USH1F	*PCDH15*	*pcdh15a* *pcdh15b*	*orbiter* *(pcdh15a)*	Protocadherin 15
USH1G	*USH1G*	*ush1g*		SANS
USH2A	*USH2A*	*ush2a*		Usherin
USH2C	*GPR95*	*gpr98*		G-protein receptor 98
USH2D	*CIP98*	*cip98a* *cip98b*		CASK-interacting protein 98
USH2`X`	*SLC4A7*	*slc4a7*		Solute carrier 4, Sodium bicarbonate cotransporter 7
USH3A	*USH3A*	*ush3a*		Clarin
USH3B				unknown

Usher Syndrome type I can result in mutations in the MY07A, USH1C, CDH23, PCDH15 and USH1G genes. Type II Usher Syndrome is caused by mutations in the USH2A, GPR98 and CIP98. The only known cause of type III is the CLRN1 gene.

Our research centers about the USH 1C gene for a couple of reasons. Firstly, USH1C is one of the genes linked to the most severe form (type I) of Usher Syndrome. With respect to the other Usher Syndrome genes, USH1C is small in size and thus encodes for a small protein. Larger proteins could be more difficult to replace because their size may disrupt the neuronal processes of other naturally existing proteins, having potentially deleterious effects on the cell. Moreover, the large size of gene products such as Usherin would make physically inserting the protein into a cell a challenge. USH1C's small size thus makes it an ideal locus of study and a prospect for gene replacement therapy.

How, why, when and where within the retina the USH1C gene interacts and ultimately gives rise to proper vision is the focus of our research of Usher Syndrome. We are contributing to a much larger effort, all the pieces of which will be needed to come to a sophisticated and complete understanding of the disorder.

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