Usher Syndrome Coalition | Using Zebrafish to Develop the First Pharmacotherapy for the Treatment of Usher Syndrome Type 1 due to Variants in MYO7A, Alaa Koleilat, Ph.D.

Hello. I would like to start off by thanking the organizers USH Connections Week for giving me the opportunity to present my work with you all today. My name is Alaa Koleilat and I recently completed my PhD at Mayo Clinic where we use the zebrafish model of Usher syndrome type 1 to explore new pharmacotherapies.

We know that Usher syndrome is characterized into three types based on hearing, vestibular, and vision impairments. However, my talk will focus on type 1 and specifically, pathogenic variance in myosin VIIA a which account for a majority of Usher syndrome type 1 cases. Myosin VIIA is a motor protein found in the stereocilia, which are these finger like projections found in the image on the right of the hair cell.

Myosin VIIA is a purple protein found in the image on the left and its role is to maintain the tension in the tipping structure, or the connecting structure between two stereocilia. When stereocilia are deflected due to sound, myosin pulls on the tip link structure-- or again, that connecting structure between the finger like projections-- allowing the mechanotransduction channel, or MET channel, to completely open causing an influx of positively charged ions into the cell.

The change in membrane potential causes a cell to depolarize and L-type voltage gated calcium channels open in response to this change in voltage, causing an increase in intracellular calcium at the bottom of the cell. Calcium then mediates the release of glutamatergic vesicles that are part of a structure called the synaptic ribbon.

This structure has a main central component called a ribeye and has a halo of glutamatergic vesicles. Glutamate is released into the synaptic cleft and binds onto the postsynaptic cell. I wanted to share some of the reasons why zebrafish is an advantageous model for the study of human disease.

There is a complete zebrafish genome available. And in fact, over 80% of genes that are known to be associated in human disease are also found in the zebrafish genome. It is fairly easy to edit genes in the zebrafish. As you can see in the image on the bottom right, we can inject DNA or RNA straight into a single cell.

Zebrafish have a high fecundity, so they are able to produce hundreds of embryos from one meeting and we can easily administer drugs straight into the water. For the purposes of studying genetic hearing loss, zebrafish are an advantageous model because the acoustic startle response is present

at five days post fertilization, so fairly quickly within their development.

An acoustic startle is exactly what it sounds like. A zebrafish exhibits a characteristic startle response to an acoustic stimulus. And in addition to having an ear with inner ear hair cells, zebrafish also have an external sensory organ called the lateral line that is composed of neural mass, the small black dots shown on the image,

that are clusters of hair cells that detect pressure and vibration. Hair cells and the lateral line are functionally analogous to hair cells in the inner ear. However, they're much easier for us to access. In 1998, an investigator named Teresa Nicholson characterized various aspects of what we're called circling mutant zebrafish, or zebrafish that were identified to swim in circles and have balance abnormalities.

Today, I'll only be talking about the Mariner mutant. She identified that the Mariner mutant has a stereocilia bundle defect, meaning an abnormality in those finger like projections at the top of the cell. This zebrafish line also does not present with a startle to a vibrational tapping stimulus.

However, they did show that neurons were activated in response to vibration or sound through quantifying the calcium signal. Now, the value shown in the far right of this table, 9 plus or minus 8% for the calcium signal, is not close to what we're seeing in the wild type animals of 100%.

However, what's important to take away is that it's not completely silent. This is quite important for the rest of the talk to understand that the zebrafish line mentioned here, the Mariner mutant, is not starting at a zero response. Two years later, Sylvain Ernest identified that indeed this zebrafish model, the Mariner mutant, has a premature stop codon early in the gene of myosin VIIA. Meaning that the myosin VIIA protein will not be produced.

For now on, I'll refer to the zebrafish line as a myosin VIIA mutant. They also demonstrated through confocal imaging the abnormal stereocilia bundle, so those finger like projections at the top of the cell. In the left of this image, you'll see wild type hair cells. The stereocilia are structured and organized.

Whereas on the right, the myosin VIIA mutant stereocilia are splayed like split ends and they aren't as organized. However, it's reported that only 36% of the hair bundles are splayed in this manner, meaning that more than half of the hair bundles are still intact. This now led us to ask what the other components of the hair cell look like in the zebrafish model.

The main component we were interested in exploring is the ribbon synapse. Again, it's a structure found at the basal end of the hair cell that is mostly composed of a central component ribeye, this large red dot here, with a halo of glutamatergic vesicles tethered to it all around.

CTBP2 is the gene that is spliced to produce this protein called ribeye. In fact, an investigator at Wash U, Dr. Lavinia Sheets, in 2011 identified that the central protein ribeye is required for L-type voltage-gated calcium channels to localize and for innovation of the post synaptic cell.

We use transmission electron microscopy in order to capture images of the ribbon synapse. We identified that the myosin VIIA mutants in the top right part of the image have the machinery necessary to produce a complete synaptic ribbon with a ribbon density and a halo of glutamatergic vesicles. And not only is the structure generated, it is also localized to the synapse.

This was not known before and is important because not only is the machinery necessary for the structure produced properly, it's also found in the appropriate location in the cell. We went on to quantify two aspects of these images, the area of that central component-- ribeye-- and the number of vesicles in the halo.

We identified that, although the ribbon area is comparable between the two groups, there were fewer tethered glutamatergic vesicles in the MYO7A mutants. We also looked at the ribbon structure using another technique, which actually labels the protein. In this case, we labeled CTBP2 which is, again, the gene alternatively spliced to produce ribeye, the central component of the synaptic ribbon shown here in this image on the top left in red.

We identified that the MYO7A mutants have both fewer ribbon containing cells and that's defined as a cell that has at least one red dot. And also, a fewer total of CTBP2 puncta across each cluster of hair cells. Now as I was going through these images, I started to notice that some of the hair cells-- or in these images here, some of the blue dots-- have one red dot or they have two red dots, et cetera.

And I was curious to know if there is a difference in the distribution of the red dots or the CTBP2 puncta. And I found that, indeed, there is a difference between wild type and MYO7A mutants. I found that a majority of wild type hair cells contain three CTBP2 puncta in the cells, so about three red dots.

And in the MYO7A mutants, majority of hair cells have two red dots, or two CTBP2 puncta. This is interesting because the ribbon synapse is essential for the transfer of sound information. Therefore, we can conclude that this differing distribution of CTBP2 may be contributing to the deafness phenotype.

As I mentioned previously, the zebrafish line was originally characterized as a circling mutant. Therefore in collaboration with Dr. Mark Masino at the University of Minnesota, we recorded videos of fish responding to a stimulus in order to quantify their swimming behavior. We calculated the turning angles of fish during their swimming episodes.

These are two videos representing the swimming behavior

from one swimming episode. On the left, you have a wild type animal responding to this stimulus. And you'll see that it's upright in an upright position with normal balance, and it tends to swim to the perimeter of the well.

The MYO7A mutant on the right is laying on its side due to the balance abnormalities and has this very tight circular swim, which is very different from the swimming behavior of the wild type animal. In this figure, we are displaying the quantification of the turning angles from a population of wild type animals and mutant fish.

On the x-axis, you have turning angle in degrees. And on the y-axis, you have cumulative relative frequency. So if we're looking at the solid black line representing the turning angles of the wild type animals, you see that about 50% of the turning angles fall around 250 to 300 degrees. However for the MYO7A mutant, the turning angle for 50% of the turning angles are at around 450 degrees.

This is a statistically significant difference and it is the first quantitative assessment of the MYO7A swimming behavior. Now, I'll present the second half of this project in which we ask the question of whether modulating the L-type voltage gated calcium channel by the use of drugs can change the phenotypes in a MYO7A mutant.

We hypothesized that we can reconstitute a functional response to sound by increasing the sensitivity of the L-type voltage-gated calcium channel through treatment with agonists or drugs that activate channels, thus augmenting any residual signal in the MYO7A mutant hair cells. This is an illustration of this hypothesis.

In panel A on the far left, we're seeing a normal hair cell that I described previously. You have myosin VIIA at the top of the stereocilia, those finger like projections, that allows the mechanotransduction channel to completely open. This is the first step in transferring a mechanical signal, the sound, into a chemical signal and thus, the way in which our hair cells communicate to the auditory nerve.

In panel B, the middle panel, we're demonstrating our hypothesis of what is happening in the hair cell

in the MYO7A mutant zebrafish. Due to the lack of a functional myosin VIIA protein, the mechanotransduction channel is unable to gape properly, so positively charged ions are unable to enter the cell.

And the first step of this process is not reached a L-type voltage gated calcium channels do not open at the bottom of the cell, thus limiting the hair cells communication with the auditory nerve. We hypothesized that treatment with L-type voltage gated calcium channel agonists, or activating compounds, increases the probability that the L-type voltage gated calcium channel will open and would increase the likelihood that if any mechanic transaction occurs, it would be sufficient to allow the release of glutamatergic vesicles in response to sound. And this is what we're seeing in panel C.

The three drugs we chose are listed here. It is important to understand the differences in these drugs in order to draw conclusions from our results. Plus minus Bay K is a potent L-type voltage gated calcium channel agonist that is known to only target the specific channel we're interested in.

It has only been used in animal models. Therefore, we were interested in moving laterally to find drugs that are accessible to human patients. The second drug we looked at, is a cognitive enhancer that activates L-type calcium channels. Neurotrophic agents, such as Nefiracetam, can also influence multiple other pathways.

Nefiracetam has been used in clinical trials to treat post-stroke apathy in adult patients. R-baclofen is an FDA approved drug to treat muscle spasticity in children. Its main mechanism of action is actually on a different receptor or channel called a GABA receptor. However, it's been reported that GABA receptors modulate various voltage gated calcium channels.

Specifically, it was identified that there is a direct interaction between the specific voltage gated calcium channel we're interested in and the GABA receptor that this drug targets. And that activation of GABA B receptor increases L-type voltage gated calcium channel currents.

Through transmission electron microscopy, the same technique I presented earlier, we were able to observe effects of these compounds on the synaptic ribbon ultra structure. And what you'll notice here, again, that even with the drug compounds, there is the synaptic machinery necessary to produce the central component ribeye and a halo of glutamatergic vesicles.

And not only are they produced, but they're still localizing to the synapse. We identified that neither plus minus Bay K nor nefiracetam had any effect on the ribbon area. And this is shown here where these statistics indicate that there is no statistical significance between these values and that the

increase--

However, we did see an increase upon incubation with R-baclofen and that this increase is statistically significant between untreated animals and treated animals, and it's indistinguishable from wild type. So the zebrafish treated with R-baclofen have ribbon areas that are exactly comparable to wild type. And the same holds true for the number of tethered vesicles.

So the number of vesicles in that halo around the central component ribeye are indistinguishable from wild type upon incubation with R-baclofen. We also assess the synaptic ribbon by, again, labeling with CTBP2 an antibody. To remind you, CTBP2 is a gene alternatively spliced to produce the principal protein component of synaptic ribbons.

As I showed you earlier, a majority of MYO7A mutant untreated hair cells have two CTBP2 puncta, or two red dots. However, upon incubation with all three compounds, there is a shift so that a majority of hair cells have three CTBP2 to puncture or three red dots per hair cell. This is an important finding because it shows that the hair cells are responding to pharmacologic treatment in the regulation of CTBP2, which is essential for the transfer of sound information to the auditory nerve.

We also assess the swimming behavior of these animals upon incubation with the three drugs. And what we're displaying here are the turning angles. On the x-axis, you have turning angle and on the y-axis, you have the cumulative relative frequency. Now if you're looking at the dotted black line-- which is a little bit difficult here to see, but it's the line farthest to the right.

You can see again that 50% of the turning angles are close to 500 degrees. And you'll notice that with treatment of nefiracetam and R-baclofen-- so the blue and purple lines-- the curve moves modestly closer to the solid black line, or the turning angles from the wild type animals. However upon incubation with plus Bay K, this green line, we see a shift-- a very robust shift that's pretty much overlapping with the wild type turning angles.

And this is also important because we're not only observing improvements in the synaptic elements of the hair cell, but we're also seeing these improvements manifesting in the swimming behavior of these animals upon treatment. This is a statistical analysis we performed. And what you'll notice in the values to the far right, these two columns here, that there isn't a shift in the mean.

However, there is a tightness in the range of the values. But we do, again, still see the most robust response upon incubation with plus minus Bay K where there's a significant decrease in the turning angles. Another way we can view this improvement is by observing the swimming trajectories or the

paths that the fish swam during video acquisition.

What we're looking at here again are the paths of an individual fish during one video acquisition. The MYO7A mutant untreated is in the far left circle and has much more circling behavior compared to the three treatment groups to the right. You can see that the treatment groups have smoother trajectories and less circular swimming.

However, the circular swimming is not completely abolished. These are two examples of fish from one swimming assessment upon incubation with two of the compounds, nefiracetam and R-baclofen. In both videos, the fish are in a more upright position and the swimming behavior is less circular. I'll play the first video on the left.

So although there's still some circular swimming initially, there's a smoother path as the fish swims. Similarly here for R-baclofen. And again, notice that the fish are upright meaning that their balance is a little bit improved. Lastly, since this is a zebrafish model of genetic hearing loss, we were interested in assessing the acoustic startle response.

Again one of the advantages of using zebrafish for the study of genetic hearing loss. We assess the acoustic startle response by placing fish in a small dish and administering stimuli at various frequencies. We quantify the absence or the presence of an acoustic startle for each individual fish for each stimulus.

On the far left, you'll see that there is very little to no acoustic startle response in the MYO7A mutants untreated. They have very little mean response rate. We identified that incubation with plus minus Bay K resulted in the most robust response with a statistically significant difference at all frequencies in the MYO7A mutant, followed by nefiracetam, which had a more modest increase.

Although this is not exactly similar to wild type, which is the column on the far right, it is an improvement from the untreated animals that had very little to no acoustic startle response. Lastly, we did not observe any improvements upon incubation with R-baclofen.

Our study is the first to report the ribbon synapse morphology of the MYO7A mutants. We identified that compared to wild type, the mutants have decreased number of vesicles tethered to ribeye. We have decreased number of ribbon containing cells, those number of red dots and blue dots. A decreased total CTBP2 puncta per neuromast and a different distribution of those puncta, the red dots.

We were also the first to quantify the abnormal swimming behavior and identify that the MYO7A

mutants have larger turning angles as part of their swimming behavior. Our results indicate that treatment with L-type voltage-gated calcium channel agonists alter hair cell synaptic elements and improve behavioral phenotypes of the MYO7A mutants.

This is the first report of a drug treatment in the MYO7A mutant. We specifically identified that R-baclofen increases ribbon area and the number of tethered vesicles. We identified that all three compounds we used shift the distribution of CTBP2 puncta to more closely resemble wild type. And we identified that all three compounds decreased turning angles on the swimming behavior.

And specifically, nefiracetam and plus minus Bay K increase acoustic startle response. Now although these drugs are having different therapeutic effects on different aspects of the phenotype, what we are showing is the MYO7A mutants are responding to a pharmacotherapy. There's still many unanswered questions and in order for us to move into the clinic for a clinical trial, we need to be certain that the drugs are acting on the pathways that we have hypothesized.

So we need to identify the exact mechanism of action in which each drug compound is acting and which of these pathways provides a therapeutic effect. We'd like to directly assess the calcium signal in the hair cells through the L-type voltage-gated calcium channel with and without agonist treatment.

And also prior to any sort of clinical translation, we need to test our findings of the use of these compounds in the mouse model because it's a closer model to humans in terms of anatomy. Zebrafish do not have a cochlea and humans do. In this video, what we're showing is a mouse model of Usher syndrome type 1 that also has a circling behavior similar to what we saw in the zebrafish.

Before I conclude, I wanted to share with you all that I realized what I presented is a lot of preclinical research, or basic research. However, this is a type of research that lays the foundation for clinical trials, which then lead to translation into practice. It's important for us to understand at the cellular level what the consequences of a genetic variant are in order to develop therapies.

So although what I presented today is part of the T0 column on the far left, it provides significant evidence that hair cells of the zebrafish model of Usher syndrome type 1 due to variants in myosin VIIA are responding to pharmacological treatment, which has never been shown before. And this approach within itself using a drug compound is a new way of thinking about therapy for Usher syndrome type 1 patients.

I would like to emphasize that science is a team sport and there are many that contributed to this

project. I would specifically also like to thank my funding source and the Mayo Clinic graduate school. Thank you very much for your attention.