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Effects of Ultrasound on Amyloid Beta 42 (Aβ42) Mediated Neurodegeneration

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Effects of Ultrasound on Amyloid Beta 42 (Aβ42) Mediated Neurodegeneration

Honors Thesis

Sarah Byrne

Department: Biology

Advisor: Amit Singh, Ph.D.

April 2017

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Abstract

Alzheimer's disease (AD) is an age related progressive neurodegenerative disease. The exact mechanisms that lead to cell death are not entirely understood. It has been shown that accumulation of amyloid-beta-42 (Aβ42) plaques generated by mis-cleavage of amyloid-precursor-protein is the cause of neurodegeneration seen in AD. This is due to the hydrophobic nature of Aβ42 due to extra two amino acids added to the typical and naturally occurring Aβ40 in the body. These Aβ42 plaques trigger neuronal death because of the toxic nature and stress they exert on the neurons. In this study, *Drosophila melanogaster* transgenic model where human Aβ42 coding cDNA is ectopically expressed in the developing fly retina comprising of retinal neurons to study the effect of ultrasound waves. Our hypothesis is to employ ultrasound wave exposure as a possible treatment to Alzheimer's Disease. Ultrasound is a high frequency and lower energy sound wave, which may have less deleterious effect on cells in the tissue. In theory, using energy emitted from these waves would break down the plaques limiting damage due to degeneration. The wild type will be used as a control to see any side effects of the ultrasound treatment, while an AD affected fly will be used to determine effectiveness of the treatments. The goal of this project is to standardize the optimum ultrasound treatment, to observe the effects on survival rates, prevent neurodegeneration by removing or decreasing plaque damage. By varying the height, medium, time, and number of treatments, the survival rate and rescue can be tracked. Further studies using larval imaging approach can be used to see early stage effects of the ultrasound. These studies will allow testing the efficacy of commonly used treatment in sports related tissue injuries to cure inflammation and also to dislodge protein aggregations in Alzheimer's disease where accumulation of Aβ42 plaques is the hallmark.

Dedication

I would like to thank Dr. Amit Singh for guidance, feedback, and valuable learning experience as well as members of the Singh Lab and family for help and support throughout the process. I would also like to thanks Bloomington Stock Centre, Developmental Studies Hybridoma Bank, and K Cho for fly reagents. Finally, thanks to The University of Dayton's Honors Program, Biology Department, and Premedical Programs, as well as the Dean's Fellowship, Szabo Grant, and Dr. Scheltens for funding and support!

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Background

Alzheimer's disease (hereafter AD) is a progressive neurodegenerative disease that has no cure to date (Shriver *et al*., 2011; Sarkar *et al*., 2016; Alzheimer's Association). It is the 6th leading cause of death in the United States affecting every 1 in 3 people ages 65 and older (Shriver *et al*., 2011; Sarkar *et al*., 2016; Alzheimer's Association). Every 66 seconds someone in the United States is diagnosed with Alzheimer's (Alzheimer's Association). It is estimated that by 2050, more than 16 million people worldwide will be living with Alzheimer's disease (AHAF, 2012). This statistic is due to a few different changing variables. One is that people are dying of less acute illness and are therefore living longer. AD is an age related disease and as the "baby boomer" population grows older, many more people will get diagnosed with Alzheimer's. Another factor is the awareness of the disease and its accompanying symptoms.

History of Alzheimer's Disease

AD symptomatic characterization is decline in cognitive and behavioral functions eventually leading to the death of the patient. Before 1906, there was not a formal diagnosis. However when writings from the past are examined, there are similar symptoms found. For example, in Egypt and Rome in ninth century B.C. and 200 AD respectively, there were texts describing Alzheimer's. In England, there were verbal tests for forgetfulness (Plontz, 2011).

As per *Drosophila* nomenclature, gene names and symbols are italicized and after first time the names of the genes are abbreviated while protein names and symbols are written in uppercase letters (http://flybase.org/static_pages/docs/nomenclature/nomenclature3.html#1)

In the 20th century, a formal diagnosis was associated with the symptoms we now use for diagnosis. The first patient to be diagnosed with Alzheimer’s disease was a 52 year old female named Frau Auguste D, who exhibited memory loss and difficulty in talking and comprehension. She was followed for many years before passing away. The doctors who followed her case reported that she could correctly identify objects from daily life such as pens or purses but when asked who her husband was, she was unable to comprehend the question or that she had a husband (Graeber, 1997).She exhibited the classic signs of memory loss and difficulty in speech and comprehension. Post-mortem, her brain was autopsied where Dr. Alois Alzheimer and Dr. Emil Kraeplin found plaques and tangles (Shriver *et al*, 2011; Plontz, 2011). These plaques served as the classic identifiers of this disease but could be diagnosed only in post mortem tissue.

Alzheimer’s Disease Symptoms

Alzheimer’s is an age related progressive disease meaning that as the patient affected with Alzheimer’s gets older, the symptoms get worse. “In its early stages, memory loss is mild, but with late-stages Alzheimer's, individuals lose the ability to carry on a conversation and respond to their environment” (Alzheimer’s Association). This is due to the continual accumulation of the Aβ42 plaques leading to neuronal death and neurodegeneration. This neurodegeneration leads to the expression of symptoms such as memory loss, cognition problems, and difficulty in speech.

These symptoms become more severe the longer the person lives with the disease. Alzheimer's is the most common form of dementia accounting for 50 to 80 percent of the reported dementia cases. Dementia is a general term for when memory loss and cognitive abilities are impaired to the point where they interfere in daily life (Alzheimer's Association). Diagnosis is made through clinical and familial experience with short-term memory loss. This can be something as simple as family member's names. People with Alzheimer's have varying lifespans once a diagnosis is made. In severe cases, it may be as little as one year. In less severe cases some have lived as long as 25 years after with the average falling somewhere in between around 8 to 10 years (Wang *et al*, 2008). Most patients suffer other system complications such as infection, pneumonia, heart failure, or malnutrition. People with more progressed Alzheimer's need more supervision and care. This combined with the duration of the disease, it can put a lot of strain on the caregivers, emotionally and financially.

Research Efforts on Alzheimer's Disease

There has been a recent increase in AD related research due to the large number of people developing this disease as well as the socio-economic impact. In the United States alone, treatment for Alzheimer's totals about $300 billion per year with out of pocket costs for the family of a single patient being around $56,000 per year (Alzheimer's Association). This staggering amount of money doesn't even provide a cure, only treatment of symptoms for improving the quality of life of the patients. With the "baby boomers" beginning to

develop the symptoms of Alzheimer's, it is very important for continued efforts to find a cure and minimize the costs of treatment (Shriver *et al,* 2011).

Clinical Regimen for Alzheimer's Disease

Currently, there are only medications that can help alleviate the symptoms. The FDA has approved 5 drugs for treatment. These help increase communication within the brain. These drugs however only mask the symptoms but do not treat the underlying cause of Alzheimer's disease (Alzheimer's Association). Due to the decreased cognition and memory, many people suffering from AD need increased care. This helps them to remember to take medications, help maintain a safe living environment, proper nutrition, and exercise (MayoClinic, 2015). Presently, there is a drug regimen that is administered to patients, which is mainly directed towards improving the quality of life. The problem is that AD disease is detected at a later stage when the damage to neurons is high and it is irreversible due to post-mitotic nature of these cells.

Present Scenario of AD

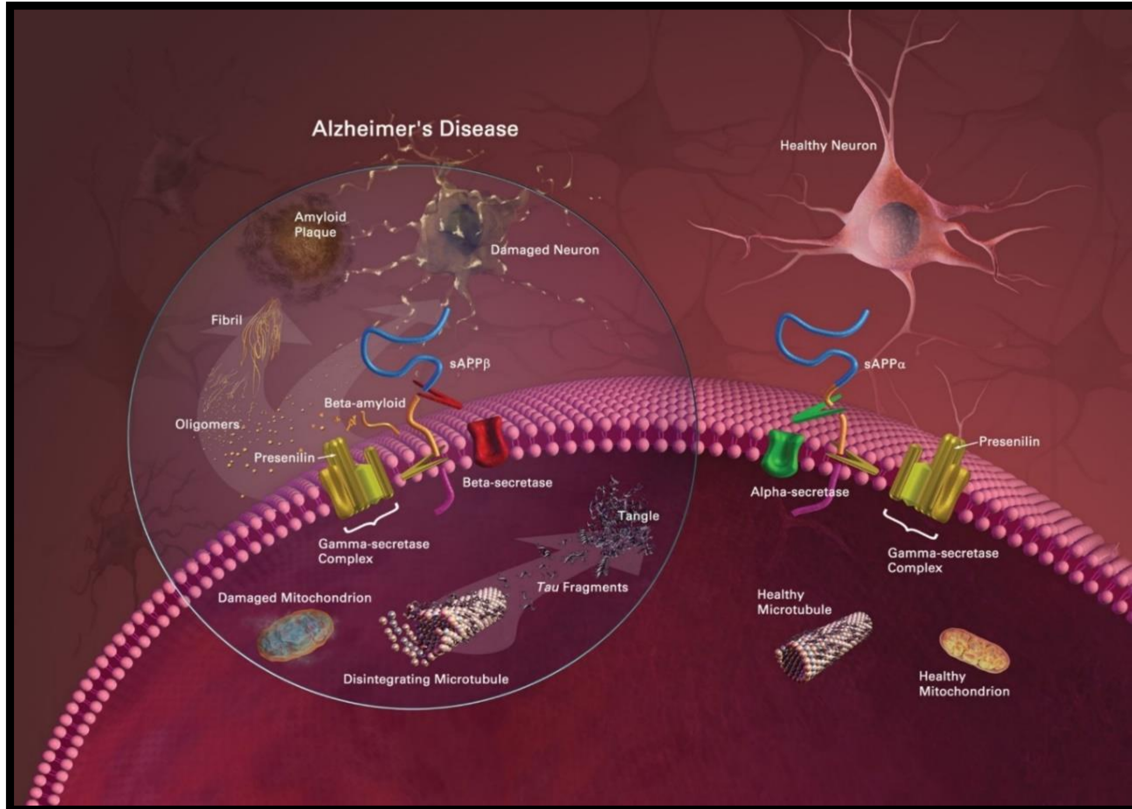

Figure 1: The Mechanism of Plaque Formation in Alzheimer's Disease. One of the main causes of Alzheimer's is the mis-cleavage by gamma secretase of the Amyloid Precursor Protein (APP), a transmembrane protein. This mis-cleavage results in an extra 2 amino acids on the protein resulting in a hydrophobic nature. These hydrophobic Aβ42 accumulate into plaques. The plaques lead to neurodegeneration [Alzheimer's Association]. (Image from http://www.nia.nih.gov/alzheimers/publication/part-2-what-happens-brainad/hallmarks-ad).

Despite the increase of research, the actual cause of Alzheimer's is still unknown. However there are many factors that are believed to trigger the onset of Alzheimer's. This includes amyloid plaques, neurofibrillary tangles, neuronal loss, oxidative stress due to Reactive Oxygen Species (ROS), and genetic basis of Apolipoprotein E (ApoE). The main

focus of my honors thesis research deals with amyloid plaques mediated neurodegeneration seen in AD. These plaques occur due to improper cleavage of a protein that forms from Amyloid Precursor Protein (APP). Typically amyloid-β-40 (Aβ40) is 40 amino acids long and is used in the nervous system so that neurons can communicate in the body (Hardy, 2009, O'Brien, 2010, Hirth, 2010, Crews, 2010). In Alzheimer's however, APP is improperly cleaved. Instead of being 40 amino acids long, it is cleaved with two additional amino acids making it 42 amino acids long (Aβ42) (Moran *et al,* 2013; Iijima,*et al* 2010; Lu, 2009; Tare *et al*, 2011). The addition of the two amino acids makes the protein hydrophobic in nature. The hydrophobic nature of the protein causes the proteins to aggregate to increase stability of the quaternary structure as well as increase the free energy of the surrounding fluid. The amyloid hypothesis suggests that accumulation of Aβ42 hinders basic cellular processes. Some of this is due to oxidative stress, mis-regulation of intracellular calcium, and ER stress (Casas-Tinto *et al*, 2011), which results in the death of neurons (Hirth, 2010). This is likely due to the progressive loss of neurons in the hippocampus and cortex (O'Brien *et al*, 2010).

The plaques are so detrimental because the human body cannot breakdown Aβ42 nor utilize this protein. When the plaques build up, the neurons can no longer communicate and die. The neuronal death is profound that the patient's brain physically shrinks to about 2/3rd the size of the typical human brain [Bier, 2005]. The focus of this project is to test ultrasound wave treatment to prevent the damage from the accumulation of the amyloid-beta (Aβ42) peptide.

Animal Models of AD

Due to the fact that the genetic and cell biological pathways are similar from insects to humans, several animal models have been employed to model AD. The mouse model is a widely employed model organism due to their close genetic and physiological similarities to humans. The mouse model is specifically useful for studying immune, endocrine, nervous, cardiovascular, skeletal and other complex physiological systems and diseases (NHGRI, 2016). Mouse models are similar to fly models in that they are low cost and quick reproductive cycle. Both can be used in experiments where a gene of interest (usually one that is encountered in humans) is altered and monitored for a resulting phenotype (Shulman *et al*, 2003; NHGRI, 2017, Emilien, 2004; Iijima, *et al*, 2004).

Drosophila: An Animal Model for AD

The fruit fly (*Drosophila melanogaster*) has been a model organism for human diseases for many years (Shulman *et al*, 2003; Emilien *et al*, 2004; Ijima *et al*, 2004; Tare *et al,* 2011). It has been shownthat around 70% of human disease genes are conserved in flies (Bier, 2005). This makes the fruit fly a useful model for studying AD. In addition to the conserved genes, *Drosophila melanogaster* is used to model neurodegenerative disease because of their genome has been completely sequenced and have a short life cycle (Bier,

2005). The life cycle of *Drosophila melanogaster* is about 12 days from egg laying to the reproducing adult. The life cycle of *Drosophila melanogaster* has 6 main stages. After fertilization, the embryo develops into a first instar larvae in about 24 hours. It takes another 24 hours for the first instar larvae to transition into the second instar. Following another 24 hours, the second instar transitions into the third instar. Next, this third instar larvae metamorphoses into pupa in around 3 days. 3-5 days later, the adult fly emerges from the pupal case. [*Drosophila melanogaster,* 2017].

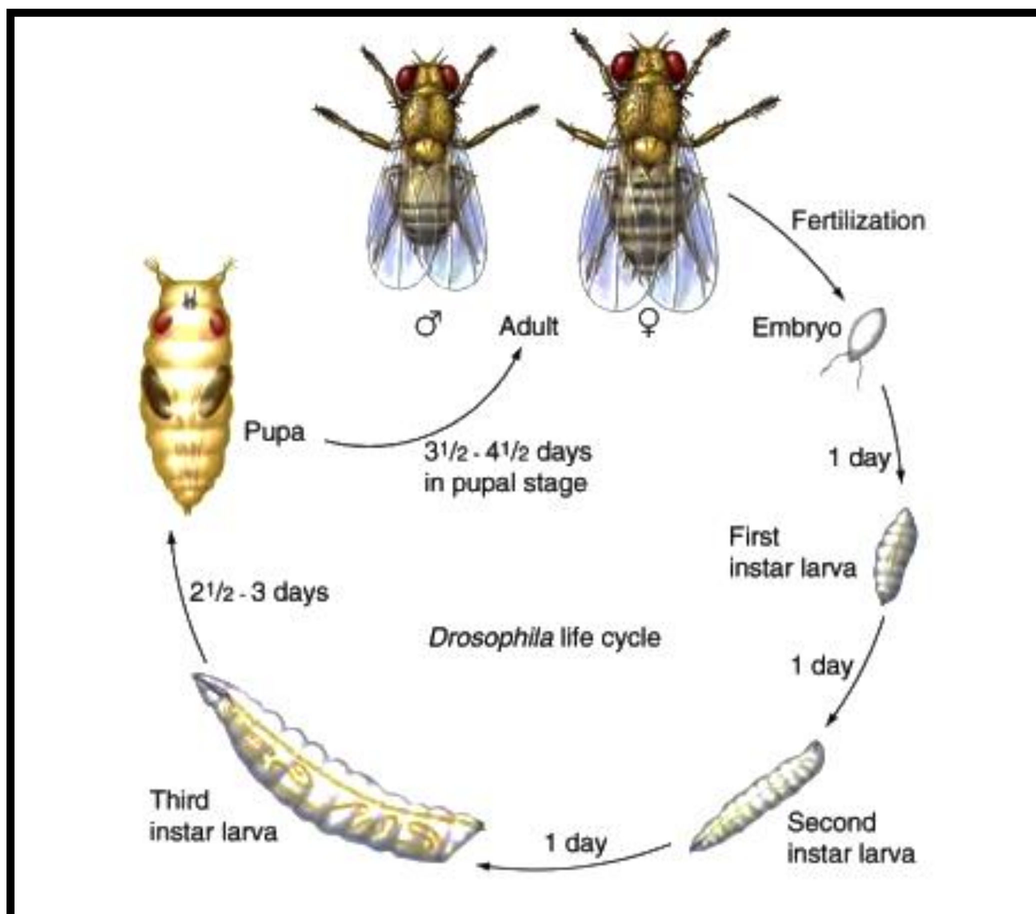

Figure 2: Life Cycle of *Drosophila melanogaster*. The life cycle of *Drosophila melanogaster* has 6 main stages. After fertilization, an embryo develops in about 24 hours into a first instar larvae. After another 24 hours, the first instar larvae transitions into the second instar. After another 24 hours, the second instar transitions into the third instar. This third instar larvae metamorphoses into pupa in around 3 days. 3-5 days later, the adult fly emerges from the pupal case. This whole life cycle takes about 12 days to complete. [*Drosophila melanogaster*, 2017].

***Drosophila* eye as disease model system**

The *Drosophila* eye (Figure 4) is an ideal organ system to assay the effect of neurodegeneration as the genes involved in eye development exhibit structural and functional similarities between insects and humans, and it is not essential for the viability or fertility of the fly (Fortini *et al*, 2000; Pandey *et al*, 2011; Rincon-Limas *et al*, 2012; Singh *et al*, 2012). *Drosophila* has a fully functional nervous system with an architecture that separates specialized functions such as vision, olfaction, learning and memory. Disease models in *Drosophila* exploit the power of its genetics (Pandey *et al*, 2011; Rincon-Limas *et al*, 2012; Bier *et al*, 2005; Prussing *et al,* 2013; Tare *et al,* 2011) its amenability to a variety of mutagenesis techniques, and its ability to express foreign genes to mimic several important neurodegenerative disorders in the compound eye (Fortini *et al*, 2000; Bonini *et al*, 2003; Hirth, 2010) In addition, *Drosophila* is amenable to chemical screens – thus allowing a quick and relatively cheap whole-animal model for testing inhibitors of the AD neurodegeneration phenotype (Gladstone *et al*, 2010; Gonsalves *et al*, 2011).

However, despite much data that is available from modeling AD in animal models such as the mouse and the fruit fly, the exact mechanism that causes Aβ42-dependent cell death has yet to be determined. Using the Gal4/UAS system on *Drosophila melanogaster* (Brand and Perrimon, 1993), we have developed an AD model with transgenic flies (Tare *et al*, 2011) that mis-expresses high levels of Aβ42 in the fly retina using a Glass Multiple Repeat (GMR) driver. GMR is a tissue specific enhancer that is expressed in the photoreceptors of the fly (Moses, 1991). When the DNA binding protein, Gal4 binds to UAS, the gene of

interest downstream is transcribed. We then can see the gene of interested expressed in the fly retina due to GMR. GMR is expressed beginning in the third instar larval stage and onward. In our cause, we have expressed human Aβ42 which is the human gene for Alzheimer's in the fly retina. Thus, our study focuses on third instar larvae, pupae, and adult flies to phenotypically identify neurodegeneration.

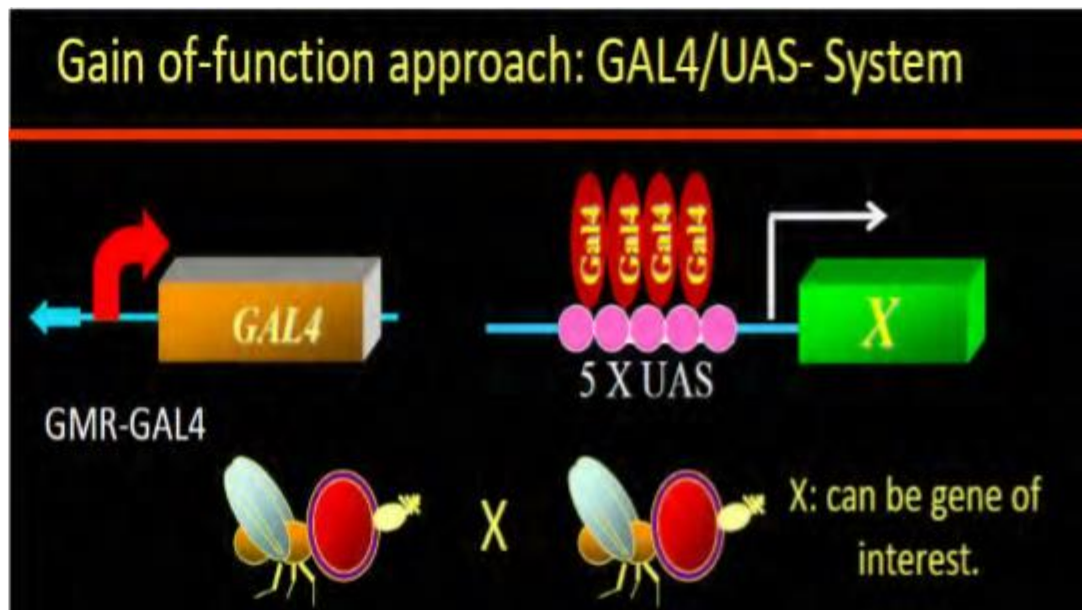

Figure 3: GAL4/UAS system for Tissue Specific Expression of Gene of Interest (Aβ42) in *Drosophila Melanogaster* eye. Glass Multiple Repeater (GMR) is a tissue specific enhancer that is expressed in the photoreceptors of the fly retina. When the DNA binding protein, GAL4 binds to UAS, the gene of interest downstream is transcribed in the domain that is targeted. In our case, due to GMR we will see the mis-expression of Human Aβ42, Human Alzheimer's, in the fly retina. (Provided by Dr. Amit Singh).

These flies exhibit progressive neurodegenerative phenotype in the retina similar to that in humans. It also produces a phenotype that when compared to the wild type adult eye and the larval eye imaginal disc showed a reduced eye size with disorganized ommatidia and bristles (Finelli, 2004). The adult *Drosophila melanogaster* eye develops

from an epithetical bi-layer structure housed inside the larvae called the eye-antennal imaginal disc giving rise to the eye, antenna, and head cuticle of the adult fly (Kumar, 2010). The imaginal discs of larvae develop into the pupal retina and then into the adult eye (Figure 4 A and B). When the plaques accumulate, the cell death is then able to be seen in the phenotype of the fly eye (Figure 4 C and D).

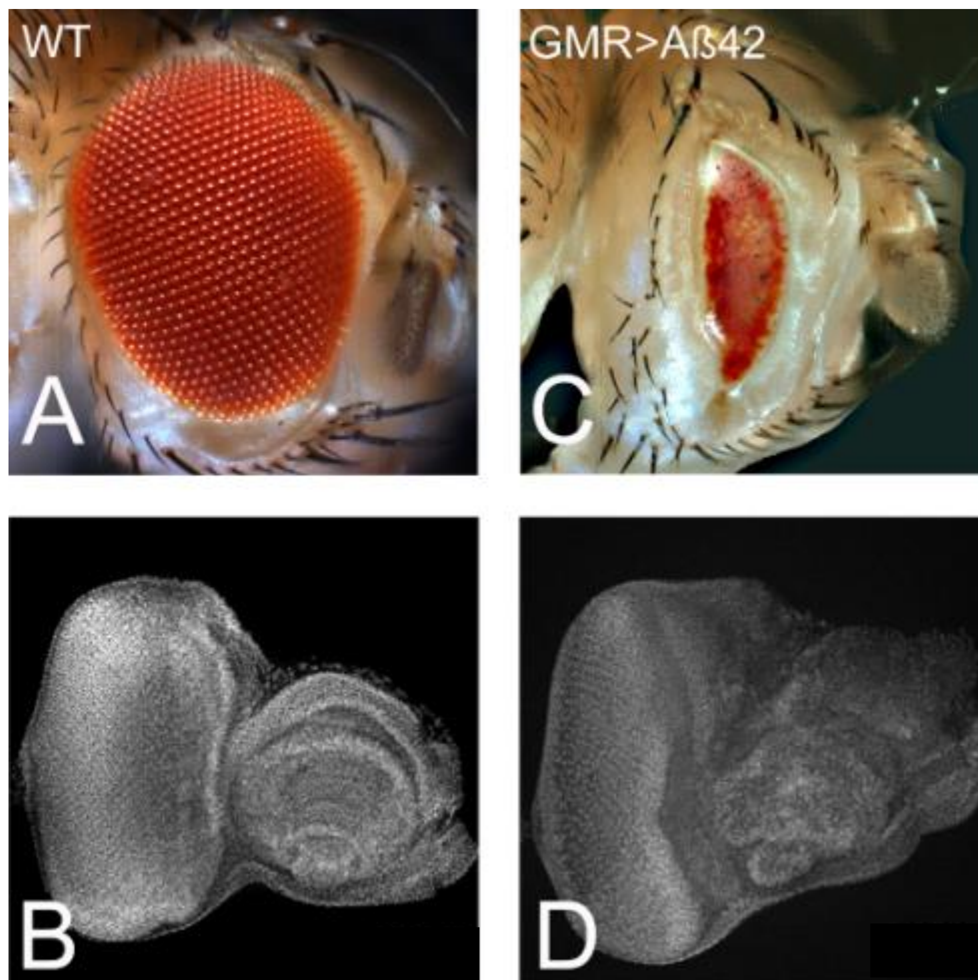

Figure 4: A: Wild Type adult eye. **B**: Wild Type imaginal disc. **C**: GMR>Aβ42 adult eye. **D**: GMR>Aβ42 imaginal disc. Neurodegeneration can be seen from the Wild Type imaginal discs and adult phenotype when compared to the GMR>Aβ42 [Cutler *et al*, 2015].

The AD phenotype can be readily seen through significant change in the adult eye structure (size, color, necrotic spots), as well as larval imaginal discs and pupal retinas. This phenotype is exhibited stronger when kept at 29°C (Tare *et a*l, 2011). As is such, for the most complete data, the cultures are maintained at 29°C. The adults however are kept at 25°C which is the most suitable for adult lifespan and reproduction. These adults of transgenic flies (GMR>Aβ42) exhibit strong neurodegeneration phenotype (Figure 4C). Similarly, in earlier stages of larval eye development, these transgenic flies eye progenitor tissue, *viz.,* eye imaginal disc exhibits neurodegeneration (Figure 4D) (Cohen, 1993). Interestingly, earlier phenotype in larval stages are weak which worsens with time as seen in adult. Thus our fly model (Figure 5) exhibits AD like neuropathology of progressive neurodegeneration (getting stronger with age).

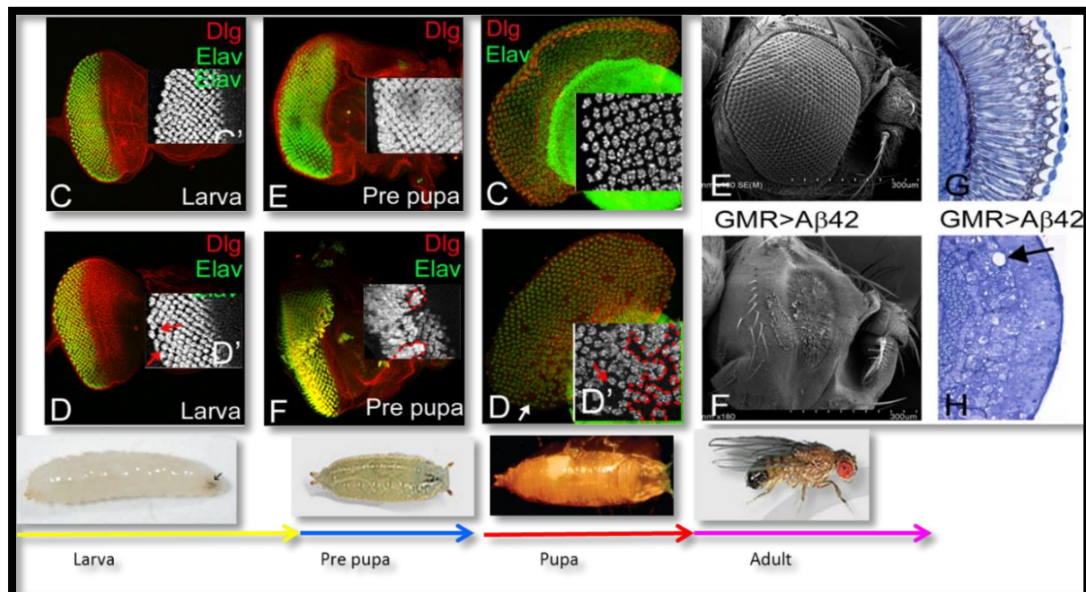

Figure 5: *Drosophila* eye model of Alzheimer's Disease phenocopies its progressive neurodegenerative disease. As the fly progresses developmentally, the GMR>Aβ42 fly (middle row) shows neurodegeneration that gets worse with time. From the imaginal discs and pupal retina, disorganization of the ommatidia and photoreceptors can be seen in the developing eye. When comparing both the Wild Type and GMR>Aβ42, the progressive neurodegeneration can easily be seen in the adult eye. (Image from Tare, *et al.*,2013).

Ultrasound: A Possible Therapeutic Approach for AD

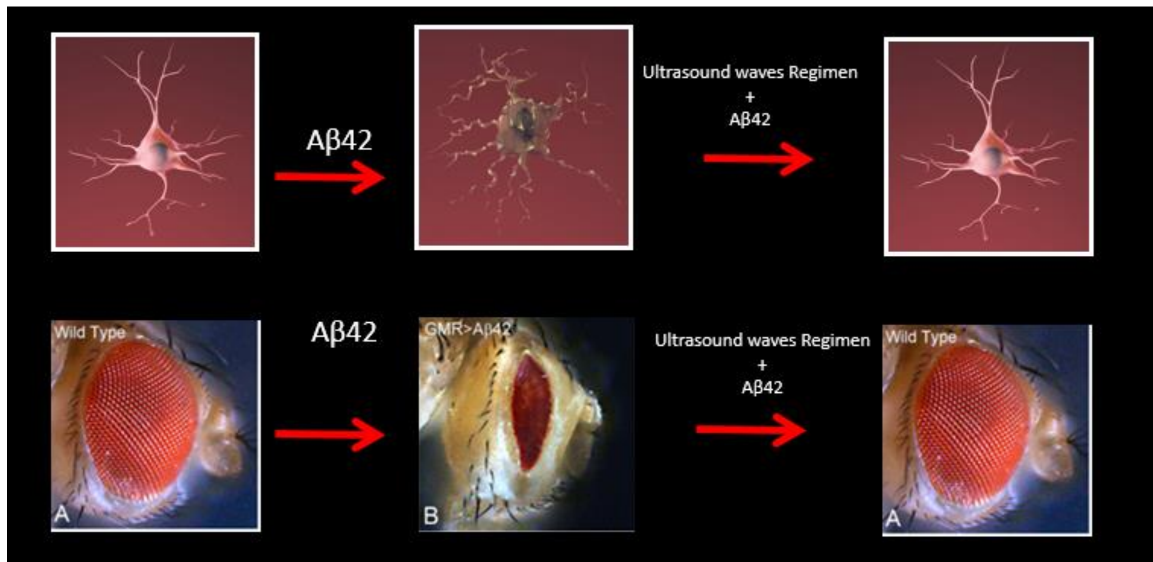

Figure 6: Can ultrasound waves dissociate amyloid plaques. Our hypothesis is that the Aβ42 plaques cause neurodegeneration. By introducing the ultrasound treatment, our theory is that the plaques will dissociate and rescue the fly retina from Aβ42 mediated neurodegeneration. (Figure provided by Dr. Amit Singh)

The aim of this project it to target Aβ42 plaques and rescue the neurodegeneration. Ultrasound has been used in many medical applications including but not limited to: diagnosis of soft tissues and organs, precision location for biopsy, imaging blood flow, bone sonography, echocardiography, 3D images or 4D (motion), and more recently, promotion of healing in tissues (Raymond *et al*, 2008; Leinenga *et al*, 2015; McLeod *et al*, 1992).

Ultrasound is a high frequency sound wave that can permeate tissue (RSNA, 2017). The proposed instrument has a frequency of 4 MHz, or a wavelength of .2 mm. Based on data from previous testing, the rate of heating with a frequency of 3 MHz was 1.19°C/min.

There was also testing done using 1 MHz which gave a 0.13°C/min rate (Hayes *et al*, 2004). By plotting and extrapolating the data, the rate is equal to .53(MHz)-.04. Theoretically, the 4 MHz ultrasound used in this experiment should emit 1.72°C/min. Using the knowledge that there is heat released as the ultrasound is used, testing can be done to determine effectiveness of ultrasound as an AD treatment. As the heat is released, there is energy that will travel through the tissues and target the Aβ42 plaques. Our hypothesis is that the energy emitted from the ultrasound as high frequency waves will disrupt the aggregation of the proteins. By breaking down the plaques, our theory is that it will prevent neurodegeneration.

The ultrasound used in this study is a Graham-Field Grafco Pocket Doppler. This instrument has a 4 MHz frequency. This frequency leads to a lower energy. The instrument was chosen to be the middle of the line for this very reason. With a lower amount of heat, there is a lesser chance that there will be damage to the flies while attempting to dissociate the aggregated plaques. This frequency however, is still strong enough with the correct dosage, to cause an effect.

Throughout this project, there will be various experiments to determine to best way to most efficiently deliver this treatment. The set up (Figure 9) will be the base to work from. All treatments will be given from above the larvae from the attached position on the clamp stand. Along the clamp stand, height can be adjusted as necessary. All trials will occur in the beaker, allowing testing to maintain consistency even when changing the media from

air to water. The time within these parameters will also be changed to identify the optimal timing for the greatest beneficial effect on the neurodegeneration. Immediately after treatment, larvae will be dissected and the eye discs will be mounted to determine efficacy of each treatment in relation to the transgenic flies (GMR>Aβ42), the wild type, and other experiments. Trials will be repeated and those treated will be allowed to hatch in an effort to measure efficacy of the treatment. Efficacy will be measured by survival rates as well as phenotypical rescue.

Materials and Methods

Fly stocks

All fly stocks used in this study can be found and described in Flybase (http://flybase.bio.indiana.edu). The fly stocks that were used in this study were GMRGal4>UAS-Aβ42 (GMR>Aβ42) (Tare et al., 2011) and Wild Type (Li et al, 2002).

This study used the Gal4/UAS system for targeting misexpression of Aβ42 in the fly retina. All experiments used the GMR line which is expressed in the imaginal discs of larvae, pupal retina, and adult eye in the fly (Moses, 1991). All stocks were kept in 25°C and 29 °C. Adult flies were kept at 25°C in which they are best suited to for lifespan and reproduction. Egg layings were kept at 29°C where the misexpression of Aβ42 in the retina exhibits a stronger neurodegenerative phenotype (Tare et al, 2011).

Immunohistochemistry

Eye-antennal imaginal discs were dissected from third instar larvae and were stained following the protocol (Singh et al., 2002). The dissected discs were washed in Phosphate Buffered Saline (PBS) and then fixed in 16% paraformaldehyde (in PBS) for 20 minute. They then were washed with PBST three times for ten minutes each totaling 30

minutes. The discs were incubated overnight in primary antibodies rat anti-ELAV (1:200) (Developmental Studies Hybridoma Bank) and rabbit Discs-large (Dlg) (1:100). The following morning, the discs were washed in PBST three times for ten minutes each totaling 30 minutes. The discs were then incubated with secondary antibodies for about 2 hour. The secondary antibodies used were: Mouse Cy3 (1:300) and Rat Cy5 (1:250) (Jackson Lab). After the two hours passed, the samples were then washed in PBST three times for ten minutes each totaling 30 minutes. The imaginal discs were then isolated from the accompanying structures. These prepared discs were then mounted in Vectashield. Immunofluorescent images were taken using the Olympus Fluoview 1000 Confocal Microscope. The final images were prepared using Adobe Photoshop[Bachmann et al, 2008].

Table of the Antibodies Used for Staining Eye-imaginal Discs.

Antibody Used	**Prepared in**	**Concentration Used**	**Source**
Primary Antibodies			
Elav	Rat	1:200	DSHB*
Dlg (Discs-large)	Rabbit	1:100	A gift from K. Cho
Secondary Antibodies			
Cy5	Rat	1:250	Jackson Lab
Cy3	Mouse	1:300	Jackson Lab

*DSHB – Developmental Studies Hybridoma Bank

Adult Eye Imaging

Using the Axioimager.Z1 Zeiss Apotome, adult eye images were able to be taken. The process to do this was as follows. The adult flies were frozen and then prepped by removing legs and wings. Then they were mounted onto a needle. This image was taken by using the extended depth focus function of the Axiovision software. This is done by compiling the individual stacks from the Z-stack. The final images were prepared using Adobe Photoshop.

Ultrasound Regimen

The Ultrasound treatments will be given using the Grafco Doppler. This probe emits a frequency of 4 MHz which is .2 mm. The probe releases 1.72°C/ min. The probe will be fixed in place to provide consistency throughout all trials. It will be attached through a clamp to a ring stand. A ruler will be attached to the ring stand in order to allow for easy checking before each trial. All trials will occur at 10 cm from the base. The samples will be placed into a 100 mL beaker to allow ample space for all samples to not be crowded together. All treatments will be timed using a stopwatch for accuracy and consistency. These times will vary depending on the study to determine an optimum time. If water is used, the amount of 2mL will be measured in a graduated cylinder. The water will be

poured over the samples after they have been placed in the beaker and under the ultrasound to minimize the amount of time under water. The treated samples were then placed into new vials of food until they hatched out and were imaged.

Results

It is an established fact that one of the reasons for onset of AD is accumulation of amyloid plaques outside the neurons in the central nervous system. The rationale of our research was to test if ultrasound waves, which are low energy waves, can dissociate the Aβ42 plaques. The larval imaginal disc Figure 4B develops into the adult eye Figure 4A. When Aβ42 is mis-expressed in the neurons of the developing eye in the GMR> Aβ42 fly, it results in neurodegeneration in the eye. We can see this neurodegeneration through the phenotype of the fly.

In an attempt to rescue from neurodegeneration, a variety of ultrasound regimes were given to trials of both wild type and GMR>Aβ42 using the setup seen in Figure 9. We needed a baseline to compare the survival rates to. This control can be seen in Table 2 and 3 for Wild Type and GMR> Aβ42 respectively. Both trials were taken through the process that the rest of the trials did with the exception of ultrasound treatment. The purpose of this was to control for any factors such as trauma due to moving from food vial to treatment beaker to a new food vial as well as stress from being in water. The Wild Type had an average survival rate of 73% and the GMR>Aβ42 had an average survival rate of 53%. These rates were then used for comparison of all other treatments and trials.

Ultrasound Treatment Regimen:

Comparing Treatment in Water to Air

To begin this study, we looked at comparing the medium through which we gave the ultrasound treatment. We compared the survival rates of both Wild Type and GMR> Aβ42 when treated through only air versus water. The amount of water that was chosen was 2 mL. This amount was selected to adequately cover the sample. However, too much water could cause drowning the flies and therefore was a factor that was considered. The 2 mL water allowed the larvae to be surrounded in water without completely covering them and thus suffocating them. In Table 1 and Graph 1 the results from this experiment can be seen. To span a range of time for comparison, 10, 15, and 30 seconds were the times selected for exposure to the ultrasound waves. From allowing the larvae of each experiment to hatch out, it was determine that for both Wild Type and GMR> Aβ42 the survival rates were higher in those that were treated in the water at all time points.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
Wild Type	0	10	30	20	0	0%
Wild Type	2	10	30	20	8	40%
Wild Type	0	10	15	20	8	40%
Wild Type	2	10	15	30	14	46%
GMR>Aβ42	0	10	10	30	4	13%
GMR>Aβ42	2	10	10	20	6	30%

Table 1: From these trials that were done on both Wild Type and GMR>Aβ42, the data suggests that the higher survival rates were seen in the flies that were treated in water.

Finding the Optimum Time Treatment

Next we wanted to determine an optimal amount of time for dosage. All flies were treated in 2 mL of water based on the results from the previous trials. For both Wild Type and GMR> Aβ42, the same treatment trials were used. Both were treated at 0, 10, 20, and 30 seconds. Results for the trials of Wild Type can be seen in Table 2 and Graph 2. The results for GMR> Aβ42 can be seen in Table 4 and Graph 4. For the Wild Type, 10 seconds had a survival rate of 33% and for 20 seconds the survival rate went up to 36%. For the GMR> Aβ42 10 seconds had a 20% survival rate and at 20 seconds this went down to 13%. Due to the slight discrepancy in the survival rates of the two times, we kept and used both times for future trials.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
Wild Type	2	0	0	30	22	73.3%
Wild Type	2	10	10	30	15	33.3%
Wild Type	2	10	20	30	11	36.6%
Wild Type	2	10	30	30	6	20%

Table 2: These trials were done on the Wild Type, the data suggests that the higher survival rates were seen in the flies that were treated for 20 seconds. Those treated for 10 seconds were very close in terms of percent survival. Due to this and the data shown for GMR>Aβ42 on times, the 10 and 20 second treatment times were kept for future trails.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
GMR>Aβ42	2	10	0	30	16	53.33%
GMR>Aβ42	2	10	10	30	6	20%
GMR>Aβ42	2	10	20	30	4	13%
GMR>Aβ42	2	10	30	30	1	3.33%

Table 3: These trials were done on the GMR>Aβ42, the data suggests that the higher survival rates were seen in the flies that were treated for 10 seconds. Due to the close survival rates in the Wild Type trials however, the 10 and 20 second treatment times were kept for future trails.

Multiple Exposures

Following this, we wanted to see if we could increase rescue of the adult phenotype by administering multiple doses of ultrasound throughout the life of the developing fly. We started treatment at the larval stage and continued to treat once per day until the first fly from the batch hatched out. Trials were run for both 10 and 20 seconds to see if one would produce a greater result than the other. As seen in Table 5 and Graph 5, the survival rates of those with multiple exposures significantly decreased. We determined that the multiple doses negatively impacted survival rate. From these trials, single dosage was determined to lead to the most effective outcome.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
Wild Type	2	0	0	30	22	73.3%
Wild Type	2	10	10	20	2	10 %
Wild Type	2	10	20	20	0	0 %
GMR>Aβ42	2	10	0	30	16	53.33%
GMR>Aβ42	2	10	10	30	1	6.66%
GMR>Aβ42	2	10	20	30	2	3.33%

Table 4. The data collected in Tables 2 and 3, the 10 and 20 second treatments were applied once daily to separate trials from the larval stage to the first adult that hatched. In both Wild Type and GMR>Aβ42, the survival rates were much lower in the multiple trials than compared to the control.

Comparing Treatment in Larval versus Pupal Stage

Finally, we tested the difference of treating larvae in comparison to treating pupae. Again, treatment was given for 10 seconds or 20 seconds in 2 mL of water as a single dose. Table 6 and Graph 6 show the results of these trials. Survival rates were slightly higher for all trials of Wild Type and GMR> Aβ42 at all times when the pupae were treated instead of the larvae.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
Wild Type	2	10	10	30 (Pupal)	15	50%
Wild Type	2	10	20	30 (Pupal)	11	36.6%
Wild Type	2	10	10	30 (Larval)	13	43%
Wild Type	2	10	20	30 (Larval)	11	36.6%
GMR>Aβ42	2	10	10	30 (Pupal)	10	33.33%
GMR>Aβ42	2	10	20	30 (Pupal)	8	26.66%
GMR>Aβ42	2	10	10	30 (Larval)	6	20%
GMR>Aβ42	2	10	20	30 (Larval)	4	13%

Table 5: From these trials that were done on both Wild Type and GMR>Aβ42, the data suggests that the higher survival rates were seen in the flies that were treated in the pupal stage in comparison to those treated in the larval stage. Both Wild Type and GMR>Aβ42 were treated at either pupal or larval stage and in separate trials were given treatment times of either 10 or 20 seconds long. All trials occurred in 2 mL of water.

Survival Rates

In addition to survival rates, imaging was taken to look at the phenotypical rescue to determine efficacy. In Figure 4, the Wild Type and GMR>Aβ42 adult eye and larval imaginal disc can be seen. The disorganization in the GMR>Aβ42 imaginal disc can be seen easily in comparison to that of the Wild Type. Similarly, the Wild Type adult eye in terms of color, shape, and size is significantly different than that of the GMR>Aβ42 adult

fly eye. In Figure 7A is the Wild Type imaginal disc after immuno-histochemical staining, the GMR>Aβ42 imaginal disc is shown in Figure 7B, and Figure 7C is the GMR>Aβ42 with ultrasound treatment imaginal disc. From this comparison we can see there is some rescues in the GMR>Aβ42 imaginal disc in terms of size and organization of the ommatidia.

Imaging

We also imaged the adult flies from some of the trials for comparison to the Wild Type adult eye and GMR>Aβ42 adult eye. In Figure 8 we can see that in both pupal treatments and larval treatments at 10 and 20 seconds some rescue can be seen in the eye. The color and shape are indicative to lesser amount of damage to the neurons of the developing eye of the GMR>Aβ42 fly. Similarly, the imaginal discs that were stained to distinguish the photoreceptors in the eye of the larvae that were treated with 10 seconds of ultrasound in 2 mL of water (Figure 7) were imaged using the confocal. This image shows increased organization of the ommatidia and less holes and larger size due to less damage from neurodegeneration. These suggest that the treatment of ultrasound is dissociating the plaques and decreasing neurodegeneration.

Discussion

Alzheimer's Studies

Alzheimer's is a widespread disease affecting close to 50 million people worldwide. It is a growing epidemic that is predicted to impact 135 million people by 2050 (Wake up world, 2017). From the increase prevalence of the disease, there has been more money and research efforts dedicated to the study of the disease, from cause to cure. Mouse and fly models have been used to study AD and some have also looked at using ultrasound as treatment. Previous studies have looked at using ultrasound waves to allow medications to pass thorough the blood brain barrier. Recently ultrasound has been used to dissociate the plaques in the brain. Studies on mice show that mice who received the treatment had significantly improved memory (Wake up world, 2017). Our studies look at optimizing the ultrasound to rescue the fly retina from neurodegeneration.

Will Ultrasound Treatment work?

Our studies looked to determine if the ultrasound waves work to dissociate the plaques in the brain leading to neurodegeneration. If the low energy sound waves emitted from the ultrasound device can break up the plaques, then rescue will be seen in the phenotype of the fly retina.

We selected the probe for this specific frequency. By using a lower energy sound wave, there is less heat emitted. This calculated heat emission is 1.72℃/min. With a lower emission of heat, there is a lesser chance of damage to the fly internally. However, the probe is still emitting sound waves at a frequency that can break up the plaques.

Our preliminary data suggests that these treatments may improve the phenotype and indicate rescue. While we have tested various dosages and treatments, the survival rates are still lower than those without testing. This indicates that while there has been some rescue, there still might be problems with the dosage. Our studies have looked into finding the optimal dosage for treatments, which would optimize the amount of rescue as well as the survival rates.

In our studies we found that using water was better than when the larvae or pupae were treated in air. We had use a quantity of water to fully cover the samples but minimal enough that the flies in pupal or larval stages can still breathe. In previous studies there was some concern that the ultrasound waves may be refracting from the surface of the glass beaker when the samples were placed in there (Raymond *et al*, 2008). By adding the water, the signal that refracts back is dampened and diffused due to the density differences. The survival rates suggest that the water does in fact lessen the damage from refracting sound waves.

Following the water study, the next study was to find the optimal timing. We needed to find a time that was long enough to create an effect but not so long to cause damage and lower the survival rate. We found by imaging the adults of those treated with 10 seconds showed a small phenotypical rescue. In an attempt to increase phenotypical rescue, we increased the dosage to 30 seconds. When we reached 30 seconds however, the survival rate decreased. We believe this is due to the heat that is emitted from the ultrasound probe. The longer the samples are subjects to the sound waves, the more heat that accompanies these waves. The survival rate suggest that the samples are able to handle the minimal amount of heat that they are exposed to during the 10-20 seconds of treatment.

Next the number of doses was considered. Keeping in mind the amount of heat released in a continuous dose, the thought was if we split up the heat released in intervals the damage would be less or removed. By spacing the treatments out by day, the samples temperature would be allowed to return to normal from any increases that occur during the treatment. However, as the results show, this is did not improve the survival rates and in fact did the opposite. The survival rates decreased significantly. This was believed to be due to a combination of stress induced from relocation for treatment as well as being continually subjected to heat from the sound waves.

Finally, we looked at the treating pupal versus larval stage. From testing it was determined that the pupal stages survived the treatment better than the larval treatment. A study showed that temperature significantly impacts juvenile mortality (Couret *et al.*,

2014). From this study and the corresponding survival rates, it would suggest that the change in temperature has a greater effect on the survival rate of the larval samples. Our data corresponds with this conclusion while still showing a rescue in the adult retina through imaging.

Optimum Dosage

Our studies suggest that ultrasound waves can dissociate the plaques that are associated with the neurodegeneration in Alzheimer's disease. This can be seen through the imaging of the imaginal discs as well as the adult eyes of those that have been subjected to treatments. While the survival rates are decrease compared to those without treatment, the optimal treatment dosage for maximal survival was determined.

After the various and repeated treatments, the optimum dosage was determined based on rescue of phenotype and survival rate. The optimum regime that our studies suggest have the highest survival rate would be as follows: a single treatment in 2 mL of water in the range of 10-20 seconds at the pupal stage of the fly's life. By doing treatment as described, the ultrasound creates the least amount of damaging side effects increasing the viability in comparison to the other trials.

This treatment was also shown through the imaging done on the adult eye to show some rescue. The rescue has not been a complete rescue when compared to the Wild Type fly. However, when the GMR> Aβ42 with treatment adult eye is compared to the GMR> Aβ42 without treatment, some rescue is seen. The color of the eye is closer to the wild-type, the size of the eye is slightly bigger, and necrotic spots are not present.

While this testing does not show complete rescue, there has been some relief of the neurodegeneration. The larval imaginal disc of the GMR> Aβ42 with ultrasound treatment does show reorganization of ommatidia and a larger area of photoreceptors in comparison to the GMR>Aβ42 without treatment. This along with the adult images suggest that ultrasound can dissociate the Aβ42 plaques to an extent providing some rescue.

Conclusions

From the tests we ran and described in detail above, we determined the most effective dosage with the highest survival rate. The optimum dosage was determined to be a single dose of ultrasound treatment, in water for 10-20 seconds at the pupal stage. As seen in the adult phenotype (Figure 8), from this treatment regimen there has been a slight rescue. This regimen combines treatments to improve efficacy while maintaining the highest survival rate.

Future Directions

Further testing and imaging should be done on the pupal retina of the pupal flies that are treated to see if the confocal imagining confirms rescue. From there, the time range of 10 to 20 seconds can try to be narrowed down to find a more specific optimum treatment. These results will be looked at by examining the adult phenotype as well as the pupal retina images. If both the adult eye and pupal retina images of the pupas that have been treated with the optimum dosage show rescue, it strongly suggests that the regimen may help reverse or block the effects of the plaques that cause the neurodegeneration in the retina of the fly.

Other imaging could be done on the wing discs to see if the ultrasound is damaging surrounding organs that could be decreasing the survival rates. If there is damage, then a mechanism could potentially be made to deliver the waves in a more specific manner to target the imaginal discs only. All of these could help find a more optimum dosage and potentially increase rescue in the retina of the fly.

Other testing is being done on other models such as mouse model have shown similar promise. The studies such as Leinenga *et al* in 2015 showed an increase of memory in 75% of the mice treated. They hope to continue these studies further on sheep and if successful, later on humans.

Data Figures

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
Wild Type	0	10	30	20	0	0%
Wild Type	2	10	30	20	8	40%
Wild Type	0	10	15	20	8	40%
Wild Type	2	10	15	30	14	46%
GMR>Aβ42	0	10	10	30	4	13%
GMR>Aβ42	2	10	10	20	6	30%

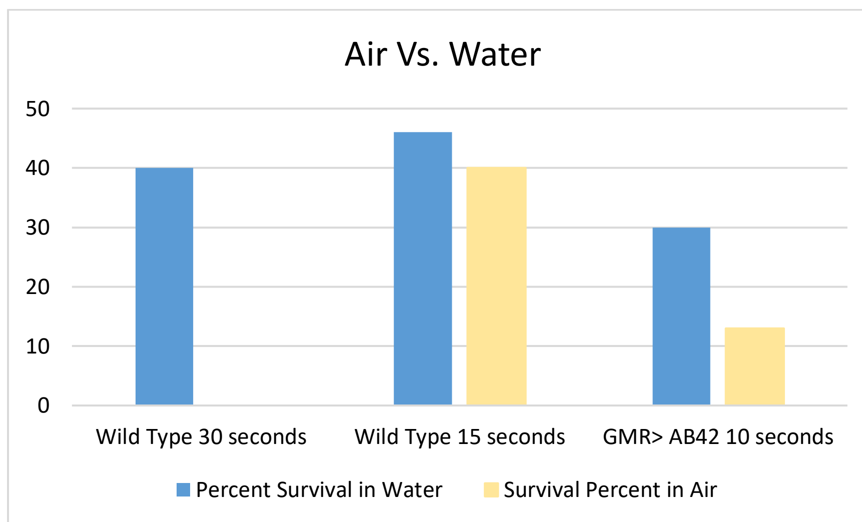

Table 1 and Graph 1: Air versus Water: From these trials that were done on both Wild Type and GMR>Aβ42, the data suggests that the higher survival rates were seen in the flies that were treated in water. The data from Table 1 was graphed (Graph 1) for visual ease.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
Wild Type	2	0	0	30	22	73.3%
Wild Type	2	10	10	30	15	33.3%
Wild Type	2	10	20	30	11	36.6%
Wild Type	2	10	30	30	6	20%

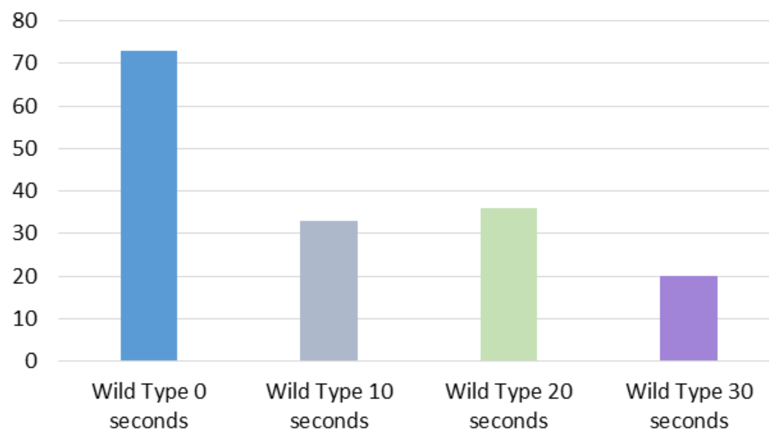

Table 2 and Graph 2: Wild Type Time Trials: From these trials that were done on the Wild Type, the data suggests that the higher survival rates were seen in the flies that were treated for 20 seconds. Those treated for 10 seconds were very close in terms of percent survival. Due to this and the data shown for GMR>Aβ42 on times, the 10 and 20 second treatment times were kept for future trails. The data from Table 2 was graphed (Graph 2) for visual ease. The top line of data from Table 2 shows the survival rate of Wild Type without exposure to the ultrasound but subjected to all other aspects of the treatment to serve as the control.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
GMR>Aβ42	2	10	0	30	16	53.33%
GMR>Aβ42	2	10	10	30	6	20%
GMR>Aβ42	2	10	20	30	4	13%
GMR>Aβ42	2	10	30	30	1	3.33%

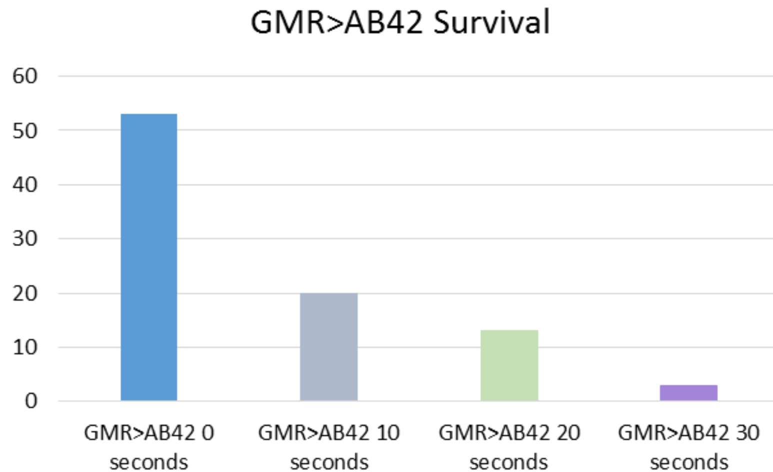

Table 3 and Graph 3: GMR>Aβ42 Time Trials: From these trials that were done on the GMR>Aβ42, the data suggests that the higher survival rates were seen in the flies that were treated for 10 seconds. Due to the close survival rates in the Wild Type trials however, the 10 and 20 second treatment times were kept for future trails. The data from Table 3 was graphed (Graph 3) for visual ease. The top line of data from Table 3 shows the survival rate of GMR>Aβ42 without exposure to the ultrasound but subjected to all other aspects of the treatment to serve as the control.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
Wild Type	2	0	0	30	22	73.3%
Wild Type	2	10	10	20	2	10 %
Wild Type	2	10	20	20	0	0 %
GMR>Aβ42	2	10	0	30	16	53.33%
GMR>Aβ42	2	10	10	30	1	6.66%
GMR>Aβ42	2	10	20	30	2	3.33%

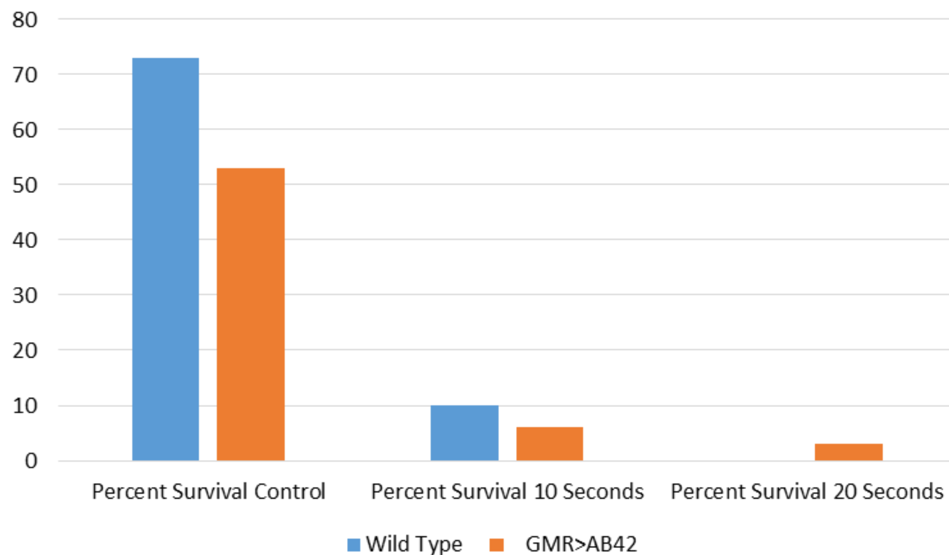

Table 4 and Graph 4: The Effect of Multiple Exposures of Ultrasound on Survival Rate: From the data collected in Tables 2 and 3, the 10 and 20 second treatments were applied once daily to separate trials from the larval stage to the first adult that hatched. In both Wild Type and GMR>Aβ42, the survival rates were much lower in the multiple trials than compared to the control. Graph 4 shows the data from Table 4 for visual ease.

Type	Amount of Water (mL)	Height (mm)	Time (seconds)	Number (initial)	Survived (final)	Percent
Wild Type	2	10	10	30 (Pupal)	15	50%
Wild Type	2	10	20	30 (Pupal)	11	36.6%
Wild Type	2	10	10	30 (Larval)	13	43%
Wild Type	2	10	20	30 (Larval)	11	36.6%
GMR>Aβ42	2	10	10	30 (Pupal)	10	33.33%
GMR>Aβ42	2	10	20	30 (Pupal)	8	26.66%
GMR>Aβ42	2	10	10	30 (Larval)	6	20%
GMR>Aβ42	2	10	20	30 (Larval)	4	13%

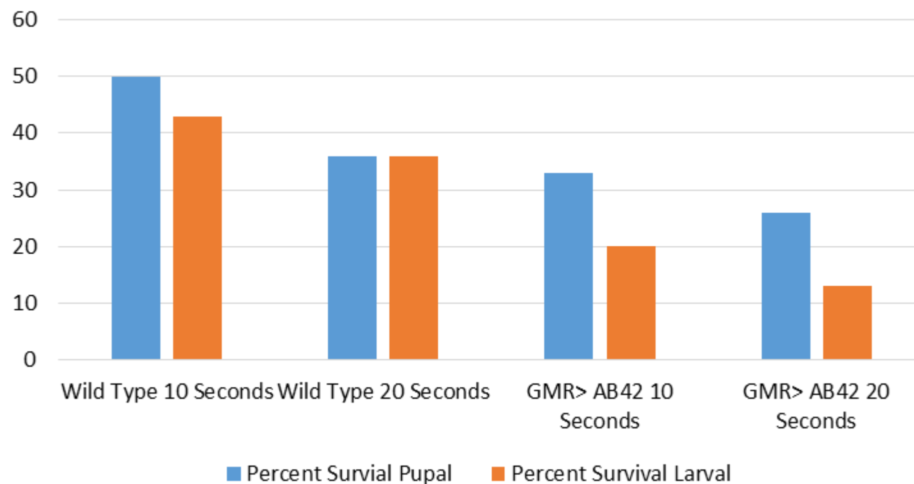

Table 5 and Graph 5: Pupal versus Larval: From these trials that were done on both Wild Type and GMR>Aβ42, the data suggests that the higher survival rates were seen in the flies that were treated in the pupal stage in comparison to those treated in the larval stage. Both Wild Type and GMR>Aβ42 were treated at either pupal or larval stage and in separate trials were given treatment times of either 10 or 20 seconds long. All trials occurred in 2 mL of water. The data from Table 5 was graphed (Graph 5) for visual ease.

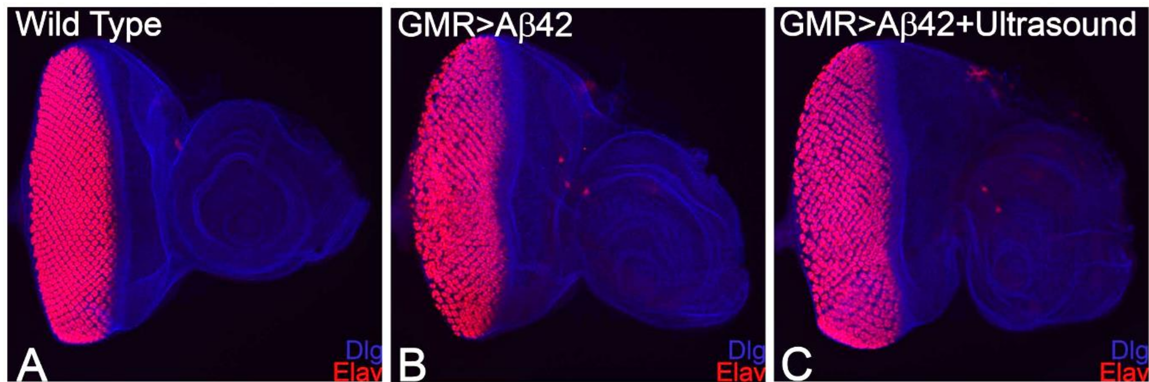

Figure 7: Comparison of the Imaginal Discs of Wild Type, GMR>Aβ42, and GMR> Aβ42 with Ultrasound Treatment. A: Wild Type larval imaging with Dlg/Elav staining **B:** GMR>Aβ42 fly larval imaging with Dlg/Elav staining **C:** GMR>Aβ42 fly larval imaging with Dlg/Elav staining after 10 second ultrasound treatment in 2 mL of water.

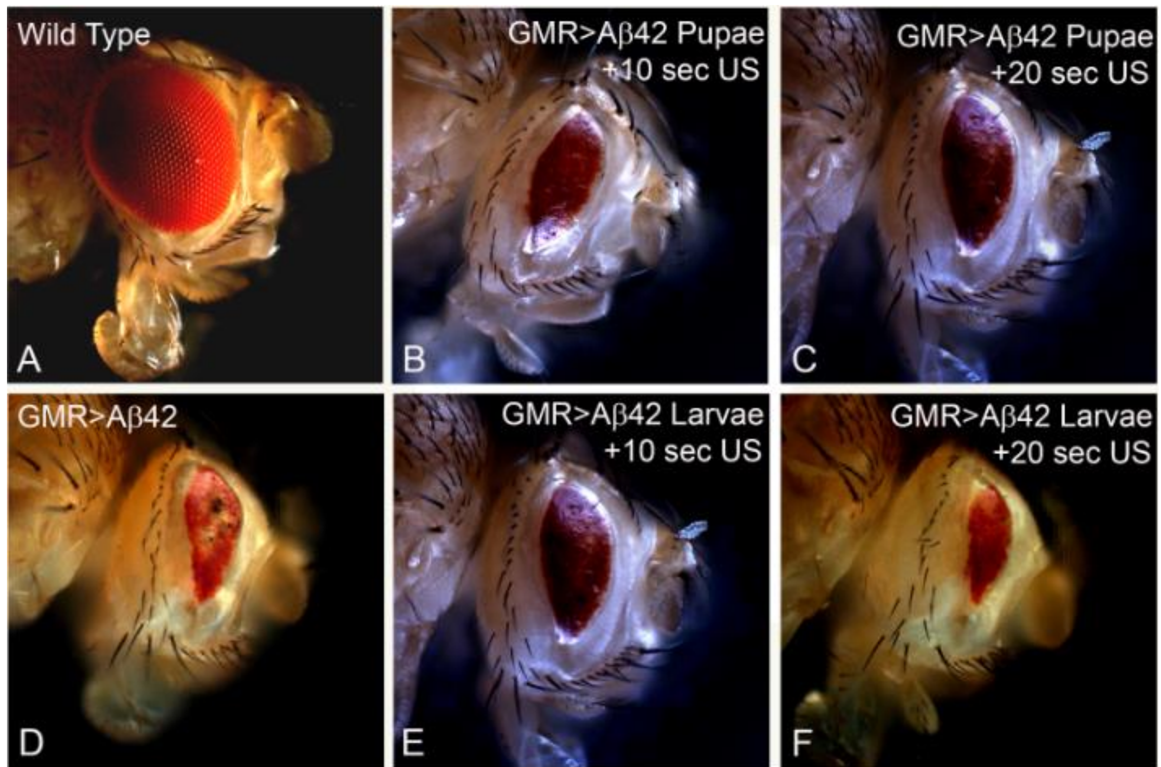

Figure 8: Comparison of Adult Eye Phenotypes in Wild Type, GMR>Aβ42, and GMR> Aβ42 with Differing Ultrasound Treatments. **A**: Wild type no treatment, **B**: GMR>Aβ42 fly 10 seconds of treatment in pupal stage, **C**: GMR>Aβ42 fly 20 seconds of treatment in pupal stage **D**: GMR>Aβ42 fly no treatment, **E**: GMR>Aβ42 fly 10 seconds of treatment in larval stage, **F**: GMR>Aβ42 fly 20 seconds of treatment in larval stage.

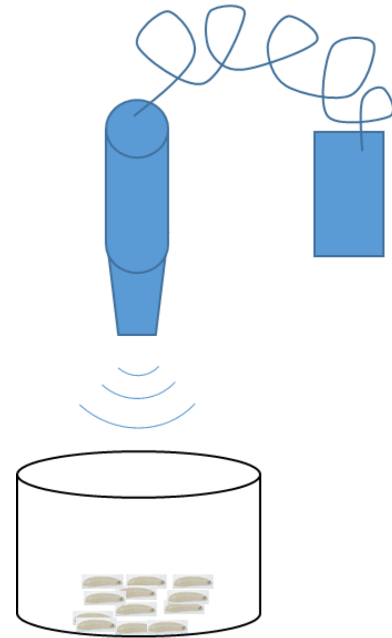

Figure 9: Ultrasound set up. All treatments and trials occurred in accordance with this protocol. The setup is shown in figure. Samples were transferred from their food vials to the beaker. The Grafco Pocket Doppler emits a 4 MHz frequency and 1.72°C per minute. The setup remain in the same place for all trials to remove factors that could affect the treatments. The height of the probe remained at 10 mm for all trails for consistency.

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