Developing Therapeutic Approaches to Neurodegenerative Diseases Associated with Defects in RNA-binding Proteins

Emily Ling and Kristine Yoon

Illinois Mathematics and Science Academy

Mentor: Dr. Jane Wu, Research Professor of Neurology and Psychiatry at the Feinberg School of Medicine at Northwestern University

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with unknown cause. However, mutations in the RNA-binding proteins, Fused in sarcoma/Translated in liposarcoma (FUS) and TAR DNA-binding protein of 43 kDA (TDP-43), have been identified in ALS patients. *Drosophila* flies expressing FUS and TDP-43 proteins in ommatidia and motor neurons were used as a *Drosophila* model of ALS. Flies were treated with 1 mg/ml of the antioxidants Vitamin C and Vitamin E, and the over-the-counter drugs Cimetidine and Naproxen. Naproxen was toxic to larvae at 1 mg/ml, but the other three drug treatments did slow down disease progression. In flies expressing TDP-43 and FUS proteins in motor neurons, treatment with Vitamin C, Vitamin E, and Cimetidine improved the larval movement index (number of peristaltic contractions in a two-minute interval) as compared to the control; however, this difference was not significant. Transgenic flies expressing wild-type TDP-43 protein in the ommatidia showed improvement after drug treatment, though this difference was not significant. Transgenic flies expressing wild-type FUS, mutant P525L FUS proteins, or mutant A315T TDP proteins in the ommatidia showed statistically significant improvement when treated with any of the three drugs. Thus, antioxidants may be potential treatments for ALS.

EXECUTIVE SUMMARY

Amyotrophic lateral sclerosis (ALS) is a disease of the nervous system, including the brain and spinal cord, in which patients gradually lose the ability to control their movement. While the exact cause is unknown, it is believed that the disease is caused by mutated proteins. Two of these proteins are TAR DNA-binding protein of 43 kDA (TDP-43) and Fused in sarcoma/Translated in liposarcoma (FUS). In many ALS patients, these proteins are mutated when one amino acid is incorrectly replaced by another. One common mutation in TDP-43 protein is called A315T, and one common mutation in FUS protein is called P525L.

In this investigation, fruit flies, or *Drosophila,* were used to recreate ALS-like symptoms. Through manipulation of the genes of the flies, their eyes and motor neurons were made to contain either the normal or mutated forms of TDP-43 or FUS proteins. Because of this, the eyes and motor ability of the flies showed damage that worsened over time. In this investigation, flies were treated with Vitamin E, Vitamin C, Cimetidine (a drug used to treat ulcers), Naproxen (a painkiller), or water as a control. Then, the eyes of each fly and crawling ability of fly larvae were observed to determine whether drug treatment could help slow down or reverse damage.

Naproxen was toxic to the flies, but the other drugs did help prevent damage. *Drosophila* flies expressing either the normal or mutated FUS protein in their eyes generally showed significant improvement over the control when treated with Vitamin C and Vitamin E, and flies expressing TDP-43 protein in their eyes also showed some improvement. In some cases, Cimetidine also helped to reduce the severity of symptoms. Also, flies expressing these proteins in their motor neurons showed improved crawling ability after being treated with Vitamin E, Vitamin C, or Cimetidine. In particular, Vitamin E nearly always helped to improve symptoms. Thus, antioxidants such as Vitamin E and Vitamin C can be potential treatments for ALS.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease characterized by the progressive deterioration and death of motor neurons. It is usually fatal within two to five years after diagnosis, with death typically caused by respiratory failure and/or paralysis (Lagier- Tourenee & Cleveland, 2009; Al-Chalabi, et al., 2012; Couthouis et al., 2012; Ugras et al., 2012). Approximately 10 percent of ALS cases are familial, and the remaining cases occur sporadically without a family history (Lagier-Tourenne & Cleveland, 2009; Ugras & Shorter, 2012). The exact cause of the disease is unknown. However, several genes and gene mutations have been identified as potential causes of the disease in both sporadic and familial cases. In ALS patients, reoccurring mutations have been identified in the Fused in sarcoma/Translated in liposarcoma (FUS/TLS, FUS) gene, and the TAR DNA binding protein of 43 kDa (TDP-43) gene (Lanson et al., 2011). These genes code for the RNA-binding proteins FUS/TLS and TDP-43, respectively (Couthouis et al, 2012).

TDP-43 is crucial for motor neuron function and regulation of gene expression (Buratti & Baralle, 2010; Fallini, Bassell, & Rossoll, 2012). Significant efforts have been put into understanding pathogenic mechanisms underlying TDP-43-associated-ALS. It remains unclear if TDP-43 proteinopathy is caused by a loss of function of TDP-43 or a gain of function toxicity, two mechanisms that are not necessarily exclusive of each other (Shiga et al., 2012). A number of mutations have been identified in the human TDP-43 gene in both sporadic and familial ALS (Kabashi et al., 2008; Rutherford et al., 2008; Van Deerlin et al., 2008; Sreedharan et al., 2008; Williams, 2009). One such ALS-linked mutation is Ala-315-Thr (A315T), is located on exon 6 of the TARDBP gene, which codes for the TDP-43 protein (Gitcho et al., 2008; Cairns et al., 2010). A315T is a missense mutation in which an alanine amino acid is replaced with a threonine amino acid at the 315^{th} position.

FUS is another RNA-binding protein involved in frontotemporal lobar degeneration (FTLD) and ALS. More than 20 mutations have been identified on the FUS/TLS gene among FTLD and ALS patients (Kwiatkowski et al., 2009; Vance et al., 2009). One of these mutations, P525L, is associated with severe juvenile ALS and familial ALS. This mutation causes cytoplasmic mislocalisation, and severely disrupts cellular functions such as nuclear import (Dormann et al., 2010; Murakami et al., 2011; Conte et al., 2012).

TDP-43 and FUS proteinopathies are a group of diseases that include ALS, as well as other neurodegenerative diseases such as FTLD, Huntington's disease, Alzheimer's disease, chronic traumatic encephalopathy, and hippocampal sclerosis (Amador-Ortiz et al., 2007; Bigio, 2008; Rohn, 2008; Van Deerlin et al., 2008; Chen et al., 2009; McKee et al., 2010; Da Cruz & Cleveland, 2011). In many neurodegenerative diseases, aggregations of ubiquinated, misfolded proteins can be found in the cytoplasm and/or nucleus of neurons. In both ALS and FTLD, TDP-43 is the primary disease protein (Neumann et al., 2006; Davidson et al., 2007; Geser et al., 2009). Also, FUS and TDP-43 are overexpressed in affected brain regions (Sieben et al., 2012). The two proteins have been shown to act together in a common genetic pathway (Wang et al., 2011). Because TDP-43 and FUS are very similar in their structure and function as RNA-binding proteins, it may be reasonable to believe that RNA processing plays an essential role in ALS and other neurodegenerative diseases (Da Cruz & Cleveland, 2011).

Studies suggest that various neurodegenerative diseases, including TDP-43 and FUS proteinopathies, are simply different manifestations of mechanisms that are similar at a molecular level (Geser et al., 2009). For example, similarly misfolded proteins with an amyloid beta-sheet conformation are found in Alzheimer's disease, Parkinson's disease, Huntington's disease, ALS, and prion diseases (Ross & Poirier, 2004). Also, as discussed above, many

neurodegenerative diseases involve the proteins FUS and TDP-43, which are very similar both structurally and functionally. Since many neurodegenerative diseases share common molecular mechanisms, the same compounds may be able to alleviate symptoms of several diseases at once.

A highly effective technique for studying the function of genes such as TARDBP and FUS/TLS is through ectopic expression of that gene in model organisms. This can be done with the Gal4 System, which allows researchers to drive expression of certain genes in specific tissues in *Drosophila* (Duffy, 2002; Southall, Elliott, & Brand, 2008). The Gal4 System involves the yeast transcriptional activator Gal4, which binds to promoters that carry Gal4 binding sites, driving the expression of the target gene (Fischer, 1988). An Upstream Activation Sequence (UAS) is a vector that consists of five Gal4 binding sites, and target genes can be subcloned into this sequence (Brand & Perrimon, 1993). When Gal4 proteins bind to the UAS, it causes the expression of the target gene. The expression of the target gene can also be restricted with drivers, which limit expression to certain tissues. This investigation used the GMR-Gal4 driver containing the glass multiple reporter (GMR) promoter, which targets the eye; it will also use the OK371-Gal4 driver, which targets the motor neurons (Li W., Li S., Zheng, Zhang, Xue, 2012).

Drosophila is a highly useful animal model for studying human diseases, with its powerful genetics, convenience in maintenance, and abundant resources for genotype-phenotype correlation. A fly takes only 10-14 days to become a mature adult from an embryo stage (Ambegaokar, Roy, & Jackson, 2010). Because it only has four pairs of homologous chromosomes, it is much easier to insert and delete genes to create transgenic organisms, as compared to mammals (Ambegaokar, Roy, & Jackson, 2010). Although flies may seem different from humans in appearance, the fundamental cellular processes and genetics are highly conserved from flies to humans, including regulation of gene expression, cell death, synaptic

transmission, and subcellular trafficking (Ambegaokar, Roy, & Jackson, 2010). This makes transgenic *Drosophila* flies ideal for the study of human genes.

Several *Drosophila* models of ALS have been created, with flies expressing TDP-43 and FUS proteins (Li et al., 2010; Chen et al., 2011; Lanson et al., 2011; Gregory, Barros, Meehan, Dobson, & Luheshi, 2012; Xia, Liu, Yang, Gal, Zhu, & Jia, 2012). Flies expressing the human FUS protein in photoreceptor cells showed symptoms of progressive retinal degeneration, including ommatidia loss, ommatidia fusion, disruption of bristles, and loss of red pigment (Chen et al., 2011). The eyes expressing the ALS-linked mutated protein demonstrated increased severity of eye phenotype (Chen et al., 2011). When expressed in the neurons of the mushroom bodies, human FUS and the ALS-mutant caused progressive loss of axons, and in the motor neurons, the protein led to swelling of the neuron cell body and degeneration of axons (Chen et al., 2011). Similarly, in a *Drosophila* model of TDP-43 proteinopathy, flies expressing human TDP-43 in photoreceptors showed signs of ommatidia loss and necrotic patches. In the motor neurons, this protein caused axon loss, protein aggregate formation, axon swelling, and neuron loss, and in the mushroom bodies, human TDP-43 caused neuron death (Li et al., 2010). These symptoms worsen over time, as is characteristic of a neurodegenerative disease like ALS. The *Drosophila* model of ALS recapitulates critical features of ALS as a human disease, sharing biochemical, clinical, and pathological characteristics (Li et al., 2010). Not only does the *Drosophila* model mimic the progressive worsening of symptoms as in ALS, it also shows neuronal loss, axon swelling, and aggregations of disease protein, which are key characteristics of ALS. The above results suggest that these transgenic flies can be used as a powerful model to screen potential therapeutic compounds. This becomes a solid foundation for us to pursue studies presented here. Based on preliminary results collected by other researchers, we propose to test

the effects of antioxidants and over-the-counter drugs in ALS-mimicking transgenic flies that express human TDP-43 or FUS.

In this study, we have begun to test different families of compounds for their effects in modifying the neurodegenerative phenotype of transgenic flies expressing human TDP-43 or FUS proteins. At the initial stage we focused on antioxidants with several over-the-counter drugs as negative controls. Antioxidants such as Vitamin E have been shown to slow down degeneration in a *Drosophila* model of optic atrophy and Parkinson's disease (Wang et al., 2006; Yarosh et al., 2008). Vitamin E has also been shown to slow down motor weakness in transgenic mice overexpressing the human tau protein (Nakashima et al., 2004). In a study of mice hippocampus neural cells, apoptosis did not occur in cells treated with Vitamin E (Choi et al., 2003); in another study, Vitamin E protected neurons from toxic amyloid beta protein (Behl, Davis, Cole, & Schubert, 1992). Vitamin E deficiency may also cause oxidative stress, so vitamin E may help prevent against oxidative stress (Shea et al., 2003). Thus, the antioxidant Vitamin E may be able to prevent or slow down the progression of neurodegenerative diseases like ALS. Another common antioxidant is Vitamin C. In a *Drosophila* model of Parkinson's disease, flies treated with Vitamin C experienced a delay in the loss of climbing ability (Khan et al., 2012). Vitamin C also has a protective effect in the nervous system (Santos et al., 2009). In addition to antioxidants, over-the-counter drugs may also have therapeutic effects with ALS. Cimeditine is a drug that is used to treat ulcers and symptoms of heartburn that are associated with stomach problems (AHFS Consumer Medicine Information, 2010). This drug has never been tested on flies. Another over-the-counter drug is Naproxen, which is a non-steroidal anti-inflammatory drug (NSAID) (Jong et al., 2008; Vlad, Miller, Kowall, & Felson, 2008). Studies

that used Naproxen on Alzheimer's disease patients showed that this drug had no statistically significant effect (Jong et al., 2008; Vlad et al., 2008).

MATERIALS AND METHODS

Drug Treatment: Drugs were crushed to a fine powder and mixed with autoclaved Millipore water to create the concentration of 1mg/mL. However, Vitamin E was first dissolved with 70% isopropyl solution for a 100 mg/ml stock solution. In each clear plastic vial, 5.6 mL of drug-containing solution and 4.7 mL of Carolina Biological Supply Company's Instant Drosophila medium were added. Autoclaved water was used as a control. For treatment, all tested flies were raised from the embryonic stage through adulthood on drug-containing food.

Drugs used: Vitamin E- Walgreen's brand, 1000 IU softgels; Vitamin C- Nature Made, 1000 mg tablets; Cimetidine- Walgreen's brand, 200 mg tablets; Naproxen- 250 mg tablets.

Transgenic flies (Crosses): For each cross, two strains of flies were used: one that carried the driver, and one that carried the target gene. Virgin female flies carrying the driver were collected, which guaranteed that all eggs would be fertilized by the males carrying the target gene. We placed 10 virgin female flies and 5 male flies in each vial.

For TDP-43 expressed in the eye, the female flies carried GMR-Gal4, which is the driver that causes gene expression in the eyes. The male flies carried either the wild type or A315T mutant version of TDP. For proteins expressed in the motor neurons, the female flies carried OK371-Gal4, which drives expression in the motor neurons. Male flies carried either wild type TDP-43, mutant A315T TDP, or the following FUS proteins: wild-type FUS4, wild-type FUS9, wild-type FUS12, and mutant P525L. The different wild-type FUS proteins result from the same genes that are incorporated into different parts of the fly genome and vary in severity of phenotype. For FUS expressed in the eye, wild-type FUS12 and P525L stocks that had been bred previously were used.

Larval Motility Assay: In order to quantify the severity of the motor neuron phenotype in *Drosophila*, a larval motility assay was performed. Transgenic fly crosses, as described above,

were cultured until larvae reached the wandering stage. At this point, larvae were carefully removed from a vial using forceps. Each larva was gently rinsed in water and placed on a black-dyed agar plate. The number of peristaltic contractions of the larval body in two minutes was counted and used as a larval movement index. The timer was started when the larva began moving forwards consistently. Two researchers observed each larva, and the two numbers were averaged for improved accuracy. At least 20 larvae were tested for each drug.

Scoring Eye Phenotypes: In order to ensure relative uniformity of the sample population, all fly vials were cleared of adult flies 24 hours before collection. Thus, any adult flies collected one day later were less than a day old and observed as Day 1 flies. Flies were put to sleep using CO_2 gas, and eye phenotypes were scored under a microscope on days 1, 3, 8, and 10. Both eyes of each fly were observed to account for any variation between the two eyes. Adult flies were transferred to new drug-containing food on each day of observation.

Eye Severity Criteria: The *Drosophila* eye consists of hundreds of tiny light-sensitive units, known as ommatidia, arranged in a highly regular grid-like pattern with a bristle between each one. *Drosophila* flies expressing TDP-43 and FUS proteins in their eyes showed significant deterioration over time. A set of eye severity criteria was created to quantify the severity of the eye phenotype.

In flies expressing the TDP-43 protein, eye severity was judged on ommatidia loss and necrosis (Li et al., 2010). Ommatidia loss is the loss of normal structure and pigment in the ommatidia. It was quantified on five levels (Fig. 1A):

Level 1: No visible ommatidia loss
Level 2: 0-25% ommatidia loss
Level 3: 25-50% ommatidia loss
Level 4: 50-75% ommatidia loss
Level 5: 75-100% ommatidia loss

Necrosis is the build-up of dead cells. It was quantified on three levels (Fig. 1B):

Level 1: No visible necrosis Level 2: Small black dots of necrosis Level 3: Large black regions of necrosis

In flies expressing the FUS protein, eye severity was judged on pigment, size, bristle organization, and necrosis. Pigment was lost from the inside out, often leading to the formation of a "red ring" of pigment at the edge of the eye. Pigment was quantified on four levels (Fig. 2B):

Level 1: Perfectly red, no pigment loss Level 2: Red ring, with some to little pigment Level 3: Red ring, with no pigment Level 4: No red ring, with no pigment

As the eye phenotype increased in severity, the size and shape of the eye became small and shrunken. Size was quantified on three levels (Fig. 2C):

Level 1: Full, round size and shape Level 2: Oval-shaped, more long than wide Level 3: Extremely small and shrunken

Disorganization of bristles increased over time and was quantified on four levels (Fig. 2D):

Level 1: Highly regular, organized bristles Level 2: 0-50% of bristles are disorganized or missing Level 3: 50-100% of bristles are disorganized or missing Level 4: All bristles are missing

Necrosis was quantified on the same three levels as in the flies expressing TDP-43 (Fig. 2E):

Level 1: No visible necrosis Level 2: Small black dots of necrosis Level 3: Large black regions of necrosis

RESULTS

Treatment Effect on FUS Transgenic Flies: In the P525L transgenic flies, all three drug treatments had statistically significant effects on the eye phenotype. There was no statistically significant difference between the eye pigment of the drug treatments and the control with a 3-way ANOVA analysis. However, Vitamin E treatment significantly improved the size ($p=0.033$) and the bristle organization ($p=0.002$) of the eyes compared to the control flies. The size of the eyes were also significantly improved by Vitamin C treatment ($p=0.006$). Additionally, Cimeditine caused a significant improvement in the bristle organization ($p=0.034$) and necrosis ($p=0.002$) compared to the control. The flies expressing the wild-type human FUS (Wt-FUS) in their eyes also showed statistically significant differences between the control and the drug treatments with a 2-way ANOVA analysis. Cimetidine worsened the eye phenotype, but Vitamin C and Vitamin E significantly improved the pigment ($p=0.01$), size ($p<0.001$), organization of bristles ($p<0.001$), and necrosis ($p<0.001$).

Treatment Effect on TDP-43-Transgenic Flies: In the eyes of flies expressing Wt-TDP-43, treatment with all three drugs improved the level of ommatidia loss; however, this improvement was not statistically significant in a 1-way ANOVA analysis. In flies expressing mutated A315T protein, all three drugs significantly improved the level of ommatidia loss ($p<0.01$), and Cimetidine significantly improved necrosis after 10 days ($p<0.01$).

Treatment Effect Over Time on TDP-43 Transgenic Flies: For flies expressing the mutant A315T protein, all three drug treatment groups, as well as the control, showed a statistically significant change in eye pigment over a period of 10 days ($p<0.001$ for all treatment groups). There was a significant correlation between the age of the flies and the treatments for Vitamin E ($p<0.001$) and the control group ($p=0.002$). For all four treatment groups of flies

expressing TDPwt5B, the age of flies was also significantly correlated to the progression of ommatidia loss ($p<0.001$ for all treatment groups).

Treatment Effect Over Time on FUS Transgenic Flies: Flies expressing wild-type FUS protein generally showed significant correlation between the age of the flies and their eye conditions. While some criteria did not change over time (Wt-FUS, bristle organization and necrosis for control), other criteria were significantly correlated with time (Wt-FUS, pigment for Vitamin C, $p<0.001$). Time also affected flies expressing P525L mutant protein in both the drug treatment and control groups. For the control group, flies showed increased severity over time in all four eye criteria ($p<0.001$ for all criteria). For all three drug treatment groups, eye condition was significantly correlated with time for all the criteria ($p<0.001$ for all groups). Figure 3 shows the effect of time on severity of eye phenotype for wild-type and mutant FUS.

Drug treatment helped to slow down progression of symptoms. When the eye phenotype severity was plotted against time in a correlation analysis, the slope represented the rate of degeneration per day. In flies expressing wild-type FUS, the slope for the eye pigment of the control was 0.025, as compared to -0.008 for Vitamin E, and 0.008 for Vitamin C. For eye size, the slope for the control was 0.025, -0.008 for Vitamin E, and -0.009 for Vitamin C. In flies expressing the mutant P525L protein, the slope for the eye pigment of the control was 0.457, but for Vitamin E it was -0.015, and for Cimetidine it was 0.0003. In general, Vitamin E and Vitamin C, and sometimes Cimetidine, slowed down and sometimes even reversed degeneration.

Larva Motility Observations: The larvae treated with any of the three drugs showed improved larva motility over the control; however, this difference was not significant for wild-type FUS 4, wild-type FUS 9, or mutant A315T proteins (Fig. 4). However, after observing the vials where the flies developed, it was observed that there was a large difference in the height

where pupae were formed. This shows that the climbing ability of larvae is dependent on the drug (Fig. 5).

ILLUSTRATIONS

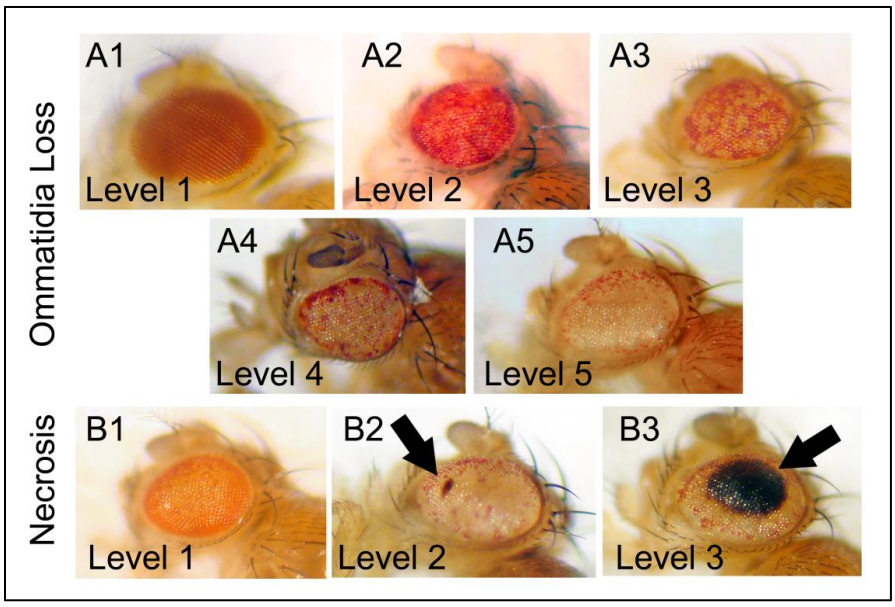

Fig. 1. Eye Severity Criteria for *Drosophila* flies expressing mutant and wild type TDP-43 in the eye. The first criterion, ommatidia loss (A1-A5), was classified into five levels: 1, No visible ommatidia loss; 2, 0-25% ommatidia loss; 3, 25-50% ommatidia loss; 4, 50-75% ommatidia loss; 5, 75-100% ommatidia loss. The second criterion, necrosis (B1-B3), was classified into three levels: 1, No visible necrosis; 2, Small black dots of necrosis; 3, Large black regions of necrosis.

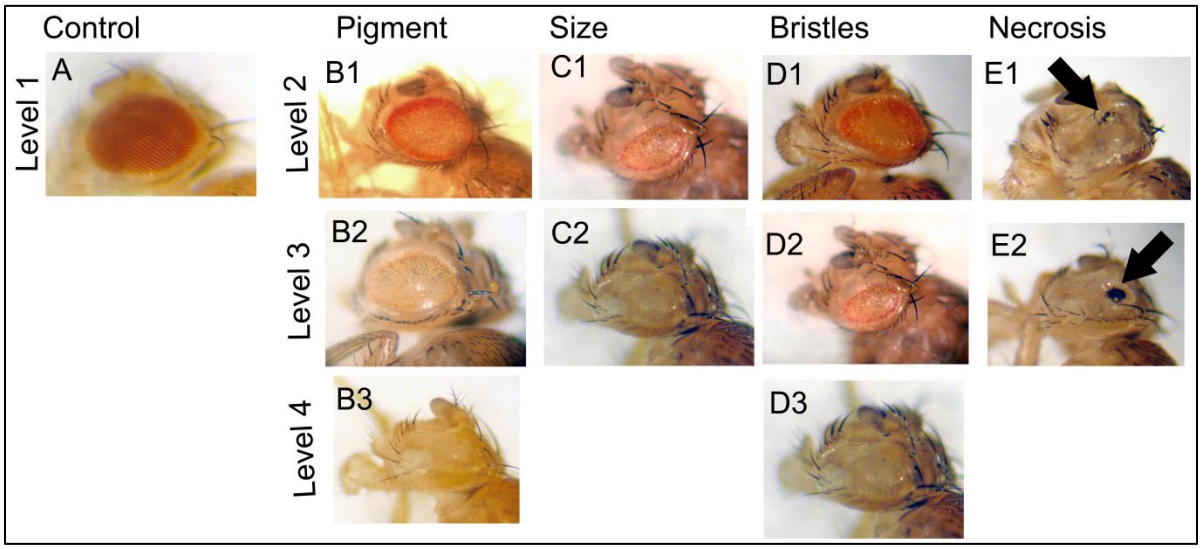

Fig. 2. Eye Severity Criteria for *Drosophila* flies expressing mutant and wild type FUS in the eye. Image A is a control fly eye, representing Level 1 for all four criteria. The first criterion, pigment (B1-B3), was classified into four levels: 1, No loss of pigment; 2, Red ring with some to little pigment; 3, Red ring with no pigment; 4, No red ring with no pigment. The second criterion, size (C1-C2), was quantified on three levels: 1, Full, normal, round eye; 2, Somewhat shrunken, oval-shaped eye; 3, Extremely small and shrunken. The third criterion, bristles (D1-D3) was classified on four levels: 1, All bristles on the eye are organized; 2, 0-50% of bristles on the eye are disorganized or missing; 3, 50-100% of bristles on the eye are disorganized or missing; 4, All bristles are missing. Finally, the fourth criterion, necrosis (E1-E2), was classified into three levels: 1, No visible necrosis; 2, Small black dots of necrosis; 3, Large black regions of necrosis.

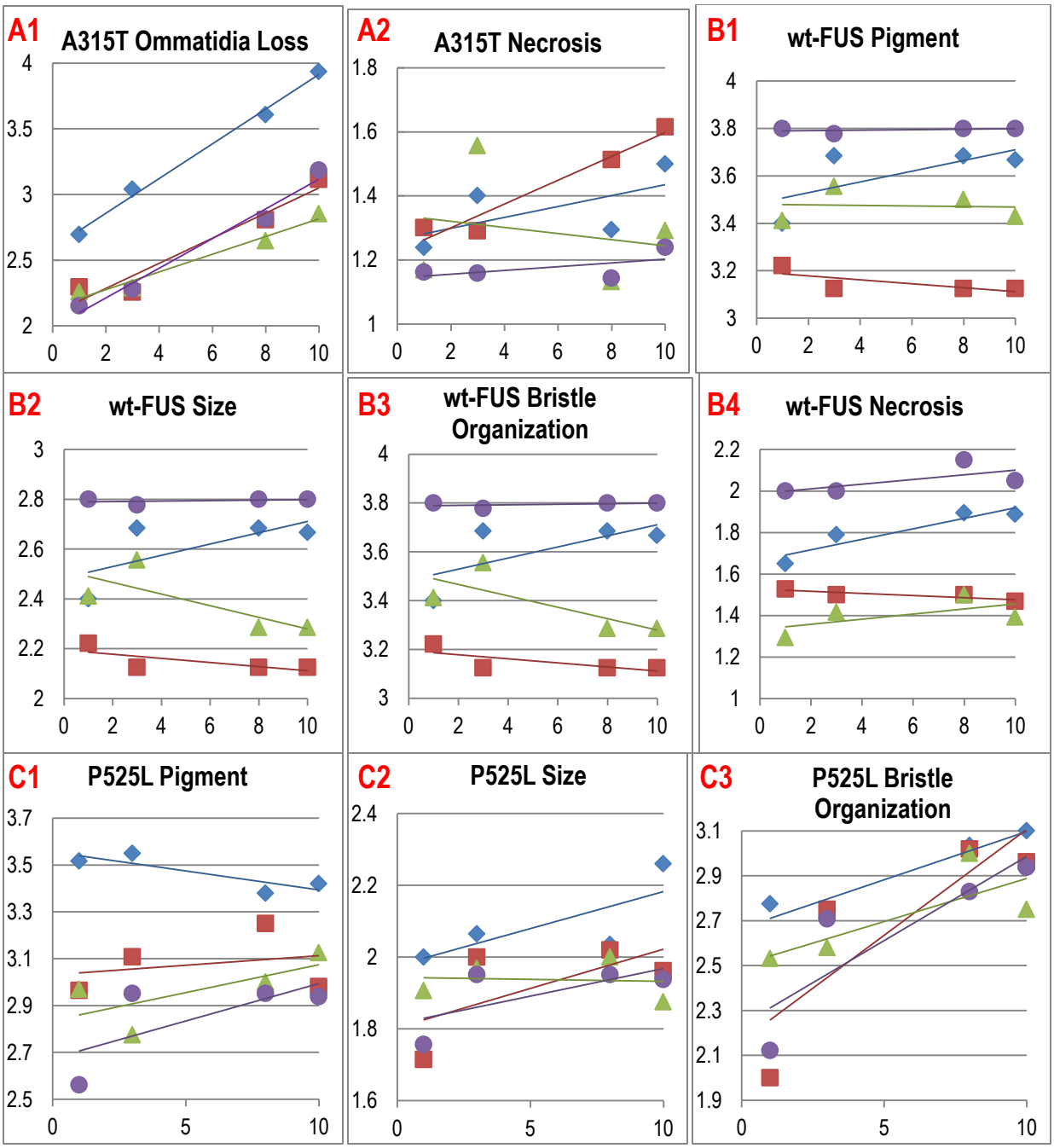

Fig. 3. Average eye phenotype severity of *Drosophila* expressing TDP-43 and FUS proteins in the eye, as it changes over time with a linear trendline. The x-axis of all graphs represents time in terms of days, and the y-axis of all graphs represents the eye severity rank for the particular criterion. In Graphs A1 and C1-C3, the trendline for the control is quite a bit higher than the trendlines of the drugs, suggesting that the phenotype has been at least partially rescued by the drugs. In graphs B1-B4, flies treated with Cimetidine demonstrated the most severe eye phenotype, followed by the Control. In most graphs, Vitamin E is at or very close to the bottom, suggesting that the eye phenotype was most rescued by treatment of Vitamin E. However, there is also some discrepancy between the different proteins and criteria. In graph A2, for example, flies treated with Vitamin E had a somewhat more severe phenotype than the other drug treatments. In general, Control or Cimetidine-treated flies demonstrated the most severe phenotype, followed by Vitamin C and Vitamin E. The average eye phenotype severity increased over time.

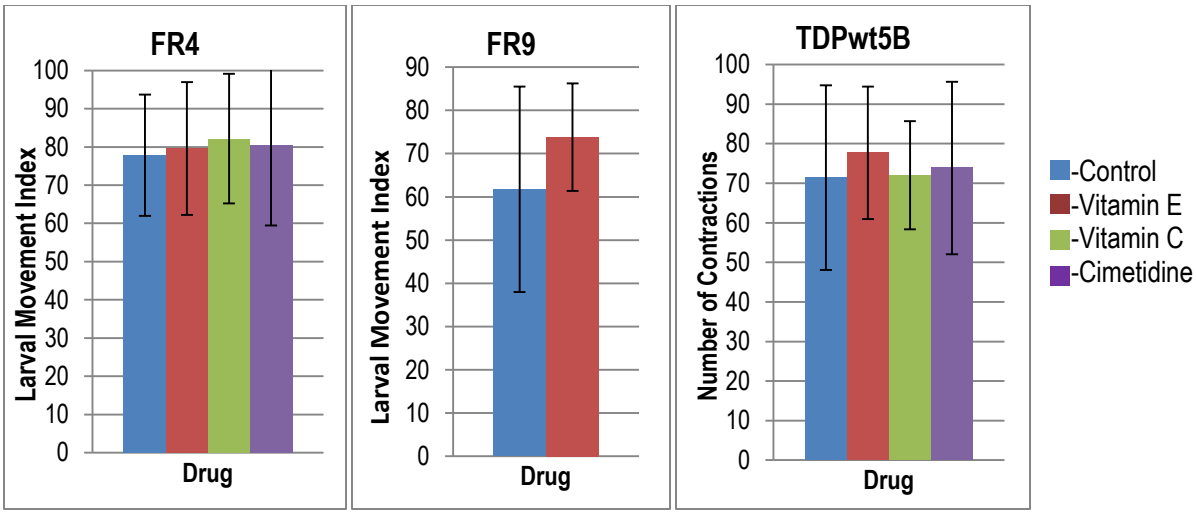

Fig. 4. Treatment of *Drosophila* with Vitamin E, Vitamin C, and Cimetidine may improve motor neuron function of wandering-stage larvae expressing TDP-43 and FUS proteins, as measured by the larval motility index, which is defined as the number of peristaltic movements of larvae in a 2-minute interval. The above graphs represent the average number of contractions for the larvae tested, with error bars of plus or minus one standard deviation. For all three ALS-associated proteins, control larvae performed fewer full-body contractions than larvae treated with any of the three drugs, suggesting that drug treatment at least slightly improved motor neuron function.

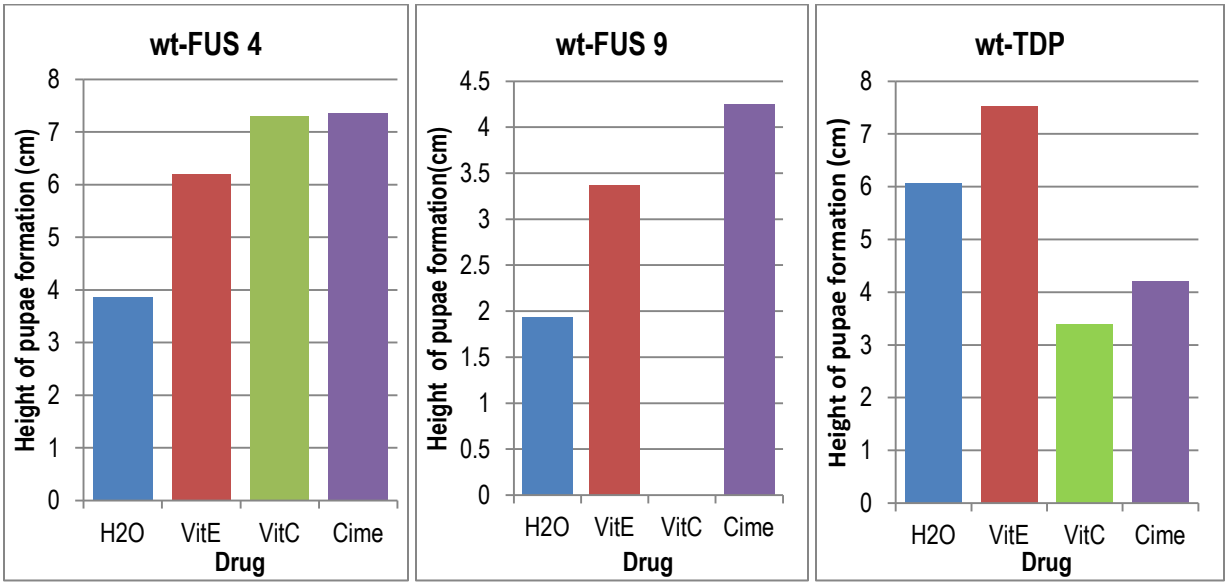

Fig. 5. Treatment of *Drosophila* with Vitamin E, Vitamin C, and Cimetidine may improve the climbing ability and motor neuron function of larvae expressing TDP-43 and FUS proteins in the motor neurons. Although many larvae were not able to eclose, *Drosophila* larvae treated with Vitamin E were able to climb up higher on the wall than control larvae. These graphs show the average height of the three highest pupae in the vials of each drug. This demonstrates improvement in motor function due to Vitamin E treatment. *Drosophila* larvae treated with Vitamin C and Cimetidine varied in the eclosion rate and climbing ability between each of the ALS-associated proteins.

DISCUSSION

Of the four drugs that were planned to be tested, there were only data for three. This is because the *Drosophila* flies treated with Naproxen died during the larvae stage and did not form pupa. This suggests that Naproxen, at 1mg/ml, is toxic to *Drosophila.*

Previous studies showed that Vitamin E is an ideal treatment for neurodegenerative diseases because it slows degeneration in *Drosophila* models of optic atrophy and Parkinson's disease (Wang et al., 2006; Yarosh et al., 2008). Our results were consistent with other studies in that Vitamin E did help to improve and slow down the progression of degeneration. Vitamin C also helped to slow down degeneration, while Cimetidine varied between improving and worsening the eye phenotype. However, these improvements for the three drug treatments were not always statistically significant.

It was found that data for most of the drugs were significantly correlated with time. In other words, the eye phenotype did worsen over time, as expected with a degenerative disease. However, the r value was very low, because the phenotype varied greatly within a single group. Nonetheless, the correlations were significant due to the large sample sizes (dF> 600 for most groups). This suggests that although individual flies may not have shown significant degeneration, as a whole, the phenotype of an entire treatment group worsened over time. Additionally, correlation analysis showed that the control group had a higher slope than Vitamin E and Vitamin C treatment groups, which generally showed a very small or even negative slope. This demonstrates that the severity of eye phenotype increased more slowly after treatment with Vitamin E or C, and drug treatment was sometimes even able to reverse degeneration.

Vitamin E, Vitamin C, and Cimetidine did improve motor neuron function in the larval motility assay. The average number of contractions was higher for the drug treatment groups as

compared to the control. However, this difference was not statistically significant due to the large amount of variation in the larva movement. The movement of the larvae was not always consistent, as some larvae changed directions multiple times, or stopped and began to burrow into the agar. Turns and burrowing did not count as full body contractions and therefore, the count may not have been accurate for all larvae. Attempts were made to increase the accuracy of the assay by counting larvae that moved consistently over the two minutes, and discarding any data from larvae that began to burrow into the agar.

Additionally, interesting results were obtained from the observations of the pupae on the vials. In *Drosophila* expressing wild-type FUS 4, larvae treated with drugs were able to form pupae much higher on the wall than the Control, and those treated with Vitamin C and Cimetidine were even able to eclose. However, in flies expressing wild-type FUS 9, there were no pupae in the vials with Vitamin C, contradicting results from a 2012 study showing that Vitamin C improves climbing ability in a *Drosophila* model of Parkinson's disease (Khan et al., 2012). However, this result could be due to toxicity of Vitamin C to the larvae, or lack of moisture in the food, which became too dry to support the development of larvae. Because *Drosophila* larvae must move away from the food to form pupae, the height of pupae formation on the walls is one measure of the larvae's climbing ability and motor neuron health. For wild-type FUS 4, wild-type FUS 9, and wild-type TDP-43 groups, larvae treated with Vitamin E were able to climb higher on the walls of the vial than the Control, suggesting a significant improvement in motor neuron function (Fig. 5).

CONCLUSION & FUTURE WORK

In this study, we used transgenic flies expressing either FUS or TDP-43 to model human ALS and focused on flies expressing the wild type and P525L mutant FUS protein, or the wild type and A315T mutant TDP-43 proteins. Further research could focus on different mutations in FUS and TDP-43 that could help create a more accurate *Drosophila* model of ALS. Other ALS-linked mutations in the FUS protein include R521G and R521C, and ALS-linked mutations in the TDP-43 protein include C9ORF72 (Suzuki et al., 2012; Sproviero et al., 2012; Smith et al., 2012). Future investigations could also study the effects of various drugs on TDP-43 and FUS proteins expressed in the mushroom bodies, in addition to the eye and motor neurons.

One way to increase the accuracy, efficiency, and volume of a drug screening test would be a high through-put system (HTS), which allows for the simultaneous study of several drugs. This method involves breeding *Drosophila* from the embryonic stage in two multi-well microtiter plates connected with a silicon adapter, with one microtiter plate having an oxygen-permeable membrane (Cagan, 2008). Not only is this technique useful for tracking the phenotype of each individual fly, but also would be less costly because it requires a small volume of drugs. While the HTS does seem efficient, removing individual flies from the wells to observe its phenotype under a microscope would be difficult. Therefore, using 1.5 mL Eppendorf tubes with punched holes on top would still have all of the benefits of the HTS, while also allowing for easy observation of specific flies. The HTS would be used to screen other types of drugs simultaneously to test for toxicity or any beneficial effect. Going forward, we will focus on 3 main categories of drugs: antioxidants, anti-amyloids, and mitochondrial protectors.

It is believed that oxidative stress and attack from free radicals may significantly damage DNA, proteins, and lipids, which is believed to play a large role in neurodegeneration (Ames,

Shigenaga, & Hagen, 1993; Cadet, 1988, Calingasan, Chen, Kiaei, & Beal, 2005). If this is true, then antioxidants could combat many neurodegenerative diseases because they help to neutralize free radicals (Uttara, Singh, Zamboni, & Mahajan, 2009). There is also evidence that the antioxidant superoxide dismutase 1 (SOD1) may play a similar role as Vitamin E in *Drosophila* models of disease (Yarosh et al., 2008). Thus, we may continue to test various doses of the antioxidants Vitamin E and Vitamin C, as well as other antioxidants such as SOD1, Vitamin A, beta-carotene, lycopene, and more.

Another possibility for therapeutic compounds is anti-amyloids. Studies have found accumulation of amyloid precursor protein in motor neurons of ALS patients, which is associated with the progression of disease (Calingasan, 2005; Steinacker, 2011). Additionally, beta-amyloid proteins have been shown to be associated with TDP-43 proteinopathy, and the presence of these amyloid proteins could trigger TDP-43 pathology (Herman, 2011). Because amyloid proteins seem to play such an important role in ALS and TDP-43 proteinopathies, anti-amyloid compounds could potentially be used to treat these diseases.

The third category of potentially therapeutic drugs is mitochondrial protectors. In a mouse model of ALS, it was found that the disease damaged mitochondrial DNA in motor neurons (Warita, 2001; Beal, 2000). The ALS-associated protein SOD1 also appears to cause mitochondrial degeneration and death in motor neurons (Higgins, 2003; Rizzardini, 2005). Mitochondrial protectors could help prevent damage to mitochondria in ALS, which could help prevent or even reverse the progression of the disease.

In addition to these three main categories, various concentrations and combinations of compounds will be tested in order to find the ideal dosage and/or combination of drugs that will most drastically slow down or prevent degeneration. The understanding of which types of

compounds are most effective will help to elucidate the molecular mechanisms underlying ALS, FTLD, and neurodegeneration as a whole.

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