Developing Therapeutic Approaches to Amyotrophic Lateral Sclerosis

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

The purpose of this investigation was to study mitochondrial protectors as potential therapies for Amyotrophic lateral sclerosis (ALS) and other neurodegenerative diseases associated with mutations in the RNA-binding proteins, Fused in sarcoma/Translated in liposarcoma (FUS) and TAR DNA-binding protein of 43 kDA (TDP-43). For ease of observation, this investigation used transgenic *Drosophila* expressing mutant FUS and TDP-43 proteins in the ommatidia of the eye. Flies were treated with food containing 5 and 10 mM of the compounds Necrostatin-1, N-Acetylcysteine (NAC), Guanabenz (GBZ), and Tacrine. The eye phenotype was observed at regular intervals using a set of criteria evaluating level of degeneration. Tacrine and Necrostatin-1 were toxic to larvae, but the other two drug treatments affected disease progression. Compared to the control, NAC and GBZ significantly improved the pupae formation, eclosion, and survival rates of the transgenic flies. In flies expressing mutant FUS proteins, NAC improved the eye phenotype compared to GBZ ($p<0.05$). Flies treated with 10 mM of NAC or GBZ often showed more severe eye phenotypes than those treated with 5 µM, suggesting that excessive dosages may worsen symptoms. Although further investigation is needed, NAC and GBZ may be potential therapies for ALS and related diseases.

Focusing Question

How do various compounds affect the rate and severity of degeneration in amyotrophic lateral sclerosis (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 made to understand pathogenic mechanisms underlying TDP-43-associated-ALS. It remains unclear if TDP-43 proteinopathy is caused by loss of function or gain of function toxicity, two mechanisms that are not necessarily exclusive of each other (Shiga et al., 2012). Several 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 ALS-linked mutation, Ala-315-Thr (A315T), is a missense mutation in which an alanine amino acid is replaced with a threonine amino acid at the 315th position of the the TARDBP gene, which codes for the TDP-43 protein (Gitcho et al., 2008; Cairns et al., 2010) (Figure 1).

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) (Figure 2).

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) (Figure 3).

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. 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). 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). This makes transgenic *Drosophila* flies ideal for the study of human genes.

Several *Drosophila* models of ALS have been developed using 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). 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. These studies suggest that transgenic flies can be used as a powerful model to screen potential therapeutic compounds. Using *Drosophila* models, we propose to test the effects of mitochondrial protectors on transgenic flies that express human TDP-43 or FUS proteins.

In this study, we will focus on mitochondrial protectors as a family of compounds that may be able to modify the neurodegenerative phenotype of transgenic flies expressing human TDP-43 or FUS proteins. These mitochondrial protectors include N-acetylcysteine (NAC), Guanabenz (GBZ), Necrostatin-1 (Nec-1), and Tacrine (Tac). First of all, NAC is both an antioxidant and a mitochondrial protector that has been shown to reverse oxidative stress in mice (Farr et al., 2003) and increase lifespan in Drosophila (Brack, Bechter-Thuring, Labuhn, 1997). In a study involving a mouse model of ALS using SOD1, NAC delayed the onset of motor impairment and increased the mouse lifespan (Andreassan, 2000). The second compound, Guanabenz, is currently available as a commercial high blood pressure drug. However, recent studies have suggested that GBZ may be effective in diseases involving the overexpression of misfolded protein. In one study, Guanabenz protected against aggregations of misfolded proteins in the cell (Tsaytler et al., 2011), and in another study, it protected against muscle degeneration in a Drosophila model of muscular dystrophy involving protein overexpression (Barbezier et al., 2011). ALS is a disease that involves the accumulation of misfolded TDP-43 and FUS proteins, so GBZ may be effective in the treatment of ALS.

The third compound tested in this investigation was Necrostatin-1, which is a necroptosis inhibitor. Necroptosis is a cell death pathway, and thus Nec-1 may be able to prevent cell death and necrosis in a disease such as ALS. Furthermore, studies have shown that Nec-1 can reduce brain tissue damage and improve motor performance in mice, demonstrating that Nec-1 has beneficial effects towards the central nervous system. The fourth and final compound used was Tacrine, which is a cholinesterase inhibitor that is commercially available to treat Alzheimer's disease (Korábečný, et al., 2012). Due to shared molecular mechanisms between neurodegenerative diseases, Tacrine may also be a potential treatment for ALS.

Materials and Methods

Materials:

Fly Crosses:

- GMR-Gal4 driver fly line
- A315T mutant TDP-43 fly lines
- FR12 mutant FUS fly lines

Fly Care and Observation:

- Fly medium
- Eppendorf tubes
- CO_2 tank
- CO_2 plates and tubing
- Microscope
- Forceps
- Camera

Drug Treatment:

- N-acetylcysteine
- Necrostatin-1
- Guanabenz
- Tacrine
- Autoclaved Millipore water
- Mortar and Pestle

- Pure Ethanol

Procedure:

Transgenic flies (Crosses)

1. Gather the necessary fly lines.
2. Place 10 virgin female flies carrying the driver and 5 male flies carrying the target gene in each clear plastic vial, covering with cotton. Fill as many vials as possible.
3. Repeat step 2 with the following combinations: GMR-Gal4 driver with A315T mutant TDP-43 and FR12 mutant FUS.
4. Allow the offspring to grow until adulthood.

Drug Treatment

1. Dilute the drugs to 5mM and 10mM by mixing the compounds with ethanol.
2. Pipette 200 µL of the solution in each Eppendorf tube containing 0.3 mL of fly food medium.
3. Defuse the solutions in the food so that the compounds are distributed equally.
4. Refrigerate when the drugs are not in use.

Embryo Collection

1. Mix 18 g of agar in 600 mL of Millipore water.
2. Add in 200 mL of apple juice.
3. Place the flask with the agar, water, and apple juice in the microwave until the solution is boiling.
4. Wait and allow the bubbles to die down
5. Pour the medium to solidify.

6. Refrigerate when not in use.
7. Add yeast paste to the middle of the agar plate to encourage fly fertility
8. Collect a 2:1 ratio of females to males of certain genotype of flies.
9. Place them in a plastic beaker with a semi permeable top for oxygen accessibility.
10. Place the agar plat on top of the beater and allow the flies to wake up.
11. Flip over the embryo collecting device and secure with rubber bands.
12. Leave in a quiet dark room for about 5 hours.
13. Remove the flies out of the embryo collecting device.
14. Collect the embryos on the agar plate with a small handheld blade.
15. Place the embryos in the food.
16. Repeat steps 7 to 15 to the following genotypes: A315T mutant TDP flies and FR12 mutant FUS flies.

Scoring Eye Phenotypes:

1. After two weeks of growth, collect the adult flies in each Eppendorf tubes.
2. Using CO2 gas to sedate the flies, observe both eyes of each fly under a microscope to account for any variation between the two eyes. Rank the severity of ommatidial degeneration using the criteria described below in “Eye Severity Criteria.”
3. Remove any pupae in the Eppendorf tube.
4. After observation, put the flies back into the tube and leave the tube upside down until the flies wake up.
5. After the flies wake up, return the tubes into its original state.
6. The first day of observation is week 1. Repeat steps 5-6 again on weeks and 3.
7. Repeat steps1-3 for each drug treatment/genotype combination.

Eye Severity Criteria**:**

In order to quantify the level of retinal degeneration in the ommatidia, a set of eye severity criteria was created. In flies expressing the TDP-43and FUS proteins, 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 (Figure 4):

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 (Figure 5):

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

Results

One of the most interesting findings was that the control group flies for both genotypes formed very few pupae and those pupae were not able to eclose. However, flies treated with N-Acetylcysteine or Guanabenz at any concentration were able to eclose. Thus, N-Acetylcysteine and Guanabenz greatly increased the pupation, eclosion, and survival rates of the flies. Necrostatin-1 and Tacrine did not have the same effects, as flies treated with these two drugs also did not eclose.

After compiling the data, the eye phenotype severities were averaged separately for each week, drug concentration, and genotype. These data were graphed against time in weeks, and linear trendlines were added. These graphs can be found in Figure 6 in the Appendix. In general, the slopes of the graphs were positive, signifying an increase in eye phenotype severity over time, which is expected with a neurodegenerative disease. However, the slope for 5 and 10 μM GBZ is negative.

To analyze the data, 2x2x2 ANOVA tests and correlation analyses were performed. A separate ANOVA test was performed to compare the eye phenotype severity of the drug and concentration combinations for each genotype. The FUS flies treated with 5 or 10 μM NAC showed a statistically significantly improvement on the eye phenotype of flies treated with 10 μM GBZ ($p<0.05$). There was no statistical significance between the drug treatments for the A315T mutant TDP-43 flies. Additionally, the higher concentrations of NAC and GBZ showed increased retinal degeneration, although this difference was not statistically significant. The correlation analyses were performed to study the relationship between time and eye phenotype severity. There was a significant correlation for most of the drug treatment groups ($p<0.05$).

Conclusion

Conclusions were drawn by observing the eppendorf tubes. The pupation of NAC, GBZ, and low concentrations of Nec-1 was about 1 to 5 flies per tube. However, in both the Tac and higher concentrations of Nec-1, there were no adult flies in any of the treatment tubes. Therefore this suggests that Tacrine and Necrostatin-1 were toxic to the flies. In addition to the lack of growth in certain treatment groups, there was no eclosion in the flies without any treatment. Thus, GBZ and NAC increased pupation and eclosion rates compared to the control, concluding that GBZ and NAC may be potential therapeutic approaches to neurodegenerative diseases.

Figure 6 shows that generally, the eye phenotypes increased as the time progressed, which was as predicted. However, the decreasing slope for 5 and 10 μM GBZ shows that these treatments may partially reverse the degeneration of phenotype, suggesting that GBZ may not only slow down the degeneration of motor neuron death in ALS, but also help treat ALS and other neurodegenerative diseases with defects in FUS and TDP-43 proteins.

In graphs A1 and B1 of Figure 6, the trendlines for the 10 mM drug concentration are higher than the trendlines for the 5mM concentrations. For example, in graph B1 the line of 10 mM of GBZ is higher than the line of 5 mM of GBZ. Similarly, in the game graph, the line of 10 mM of NAC is higher than the line of 5 mM of NAC. These results demonstrate that higher drug concentrations may have negative effects on the eye severity. In addition, the flies treated with the high concentration of Nec-1 were not able to eclose. However, the flies treated with the lower concentration of Nec-1 were able to pass the pupae stage and live into their adult stage, furthering supporting our findings that high concentrations may worse eye severity and even be toxic for the flies

Looking at the data analysis result from the ANOVA 2x2x2 tests, many conclusions coul be drawn. 10 mM of GBZ and both concentrations of NAC showed to improve the eye phenotypes, with NAC being more effective than GBZ. Thus, the results suggest that NAC and GBZ may be effective therapeutic approaches to neurodegenerative diseases with overexpression of FUS and TDP-43 proteins.

Discussion

The results suggested that N-Acetylcysteine and Guanabenz may be effective in the treatment of ALS, while Necrostatin-1 and Tacrine are not. Nec-1 and Tac yielded the same results as the control and were unable to rescue the degenerative phenotype like NAC and GBZ. This is somewhat unexpected, but it does suggest a few things about the pathology of ALS. Nec-1 is a necroptosis inhibitor, so the results suggest that the necroptosis pathway does not play a major role in ALS. Tacrine is a commercial Alzheimer's disease drug, so its ineffectiveness suggests that ALS and Alzheimer's disease do have significant differences, and Tacrine targets an aspect of Alzheimer's that is not present in ALS.

The effectiveness of NAC and GBZ was expected, as it is consistent with other scientific literature. Previous studies have shown that NAC was effective in a mouse model of ALS using SOD1 (Andreassan, 2000), and this investigation has shown that NAC is effective in a Drosophila model using TDP-43 and FUS. NAC is an antioxidant, so these results may demonstrate that oxidative stress plays a significant role in the pathology of ALS. The results for GBZ are also expected, as GBZ has been shown to be effective in diseases involving the overexpression of misfolded protein (Tsaytler et al., 2011). This investigation confirms and further supports the potential of GBZ in diseases such as ALS. Although further testing may be needed, GBZ and NAC are potential ALS treatments.

Future work relating to GBZ and NAC may include drug screening in mouse models using TDP-43 and FUS, and ultimately, clinical trials in humans. Other suggestions for future study include drug screening on Drosophila models that express disease proteins in the mushroom bodies and/or the motor neurons, which is more applicable to human ALS. The investigators are currently working on an automated larval locomotion assay that would use a

computer program to track the motion of larvae that express disease proteins in the motor neurons. This assay would provide large sample sizes and quantitative data, which is useful for drug screening. There is also much work that can be done with the methods already used in this investigation. Some suggestions include the testing of anti-amyloid drugs, as well as various combinations and concentrations of compounds. Additionally, improvements to the high-throughput system used in this investigation could improve the efficiency and sample size of the data, making this assay more effective. These future studies hold great potential for those affected by ALS.

Reference List

Al-Chalabi, A., Jones, A., Troakes, C., King, A., Al-Sarraj, S., van den Berg, L.H. (2012). The genetics and neuropathology of amyotrophic lateral sclerosis. *Acta Neuropathologic*, 124(3), 339-352.

Amador-Ortiz, C., Lin, W.L., Ahmed, Z., Personett, D., Davies, P., Duara, R.,…Dickson, D.W. (2007). TDP-43 immunoreactivity in hippocampal sclerosis and Alzheimer's disease. *Annals of Neurology*. 61(5), 435-45.

Ambegaokar, S.S., Roy, B., Jackson, G.R. (2010). Neurodegenerative Models in Drosophila: Polyglutamine Disorders, Parkinson Disease, and Amyotrophic Lateral Sclerosis. *Neurobiology of Disease*. 40(1), 29-39. doi:10.1016/j.nbd.2010.05.026.

Andreassan, O.A., Dedeoglu, A., Klivenyi, P., Beal, M.F., Bush, A.I. (2000). N-acetyl-L-cysteine improves survival and preserves motor performance in an animal model of familial amyotrophic lateral sclerosis. *Neuroreport.* 3:11 (11), 2491-2493.

Barbezier, N., Chartier, A., Bidet, Y., Buttstedt, A., Voisset, C., Galons, H., Simonelig, M. (2011). Antiprion Drugs 6-aminophenanthridine and Guanabenz Reduce PABPN1 Toxicity and Aggregation in Oculopharyngeal Muscular Dystrophy. *EMBO Molecular Medicine*. 3(1), 35-49. doi:10.1002/emmm.201000109

Bigio, E.H. (2010). TAR DNA-binding protein-43 in amyotrophic lateral sclerosis, frontotemporal lobar degeneration, and Alzheimer disease. *Acta Neuropathologica*. 116(2), 135-140. doi:10.1007/s00401-008-0405-z.

Brack, C., Bechter-Thüring, E., Labuhn, M. (1997). N-acetylcysteine Slows Down Ageing and Increases the Life Span of Drosophila Melanogaster. *Cellular and Molecular Life Sciences*. 53(11-12), 960-966.

Brand, A.H., Perrimon, N. (1993).Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development.* 118, 401-415.

Burrati, E., & Baralle, F.E. (2010). The multiple roles of TDP-43 in pre-mRNA processing and gene expression regulation. *RNA Biology*. 7(4), 420-429.

Cairns, N.J., Perrin, R.J., Schmidt, R.E., Gru, A., Green, K.G., Carter, D.,…Baloh, R.H. (2010). TDP-43 proteinopathy in familial motor neuron disease with TARDBP A315T mutation: a case report. *Neuropathology and Applied Neurobiology*. 36(7), 673-679. doi:10.1111/j.1365-2990.2010.01121.x.

Chen, Y., Yang, M., Deng, J., Chen, X., Ye, Y., Zhu, L., … Wu, J.Y. (2011). Expression of human FUS protein in Drosophila leads to progressive neurodegeneration. *Protein & Cell.* 2(6), 477-486. doi:10.1007/s13238-011-1065-7

Conte, A., Lattante, S., Zollino, M., Marangi, G., Luigetti, M., Del Grande, A., … Sabatelli, M. (2012). P525L FUS mutation is consistently associated with a severe form of juvenile amyotrophic lateral sclerosis. *Neuromuscular Disorders*. 22(1), 73-75.

Couthouis, J., Hart, M.P., Erion, R., King, O.D., Diaz, Z., Nakaya, T., … Gitler, A.D. (2012). Evaluating the role of the FUS/TLS-related gene EWSR1 in amyotrophic lateral sclerosis. *Human Molecular Genetics*. 21(13), 2899-911.

Da Cruz, S., & Cleveland, D.W. (2011). Understanding the role of TDP-43 and FUS/TLS in ALS and beyond. *Current Opinion in Neurobiology*. 21(6), 904-919.

Davidson, Y., Kelley, T., Mackenzie, I.R., Pickering-Brown, S., Du Plessis, D., Neary, D., … Mann, D.M. (2007). Ubiquitinated pathological lesions in frontotemporal lobar degeneration contain the TAR DNA-binding protein, TDP-43. *Acta Neuropathologica*. 113(5), 521-533.

Degterev, A., Huang, Z., Boyce, M., Li, Y., Jagtap, P., Mizushima, N., ... Yuan, J. (2005). Chemical Inhibitor of Nonapoptotic Cell Death with Therapeutic Potential for Ischemic Brain Injury.*Nature Chemical Biology*. 1(2), 112-119.

Dormann, D., Rodde, R., Edbauer, D., Bentmann, E., Fischer, I., Hruscha, A., … Haass, C. (2010). ALS-associated fused in sarcoma (FUS) mutations disrupt Transportin-mediated nuclear import. *The EMBO Journal*. 29, 2841-2857. doi:10.1038/emboj.2010.143

Duffy, J.B. (2002). GAL4 System in Drosophila: A Fly Geneticist's Swiss Army Knife. *Genesis*. 34(1-2): 1-15.

Fallini, C., Bassell, G.J., Rossoll, W. (2012). The ALS disease protein TDP-43 is actively transported in motor neuron axons and regulates axon outgrowth. *Human Molecular Genetics*. 21(16), 3703-3718. doi:10.1093/hmg/dds205.

Farr, S.A., Poon, H.F., Dogrukol-Ak, D., Drake, J., Banks, W.A., Eyerman, E., ... Morley, J.E. (2003). The Antioxidants Alpha-Lipoic Acid and N-acetylcysteine Reverse Memory Impairment and Brain Oxidative Stress in Aged SAMP8 Mice. *Journal of Neurochemistry*. 84(5), 1173-1183.

Fischer, J.A., Giniger, E., Maniatis, T., Ptashne, M. (1988). GAL4 activates transcription in Drosophila. *Nature*. 332(6167), 853-856.

Geser, F., Martinez-Lage, M., Robinson, J., Uryu, K., Neumann, M., Brandmeir, N.J., … Trojanowski, J.Q. (2009). Clinical and pathological continuum of multisystem TDP-43 proteinopathies. *Archives of Neurology*. 66(2), 180-189.

Gitcho, M.A., Baloh, R.H., Chakraverty, S., Mayo, K. Norton, J.B., Levitch, D., … Cairns, N.J. (2008). TDP-43 A315T Mutation in Familial Motor Neuron Disease. *Annals of Neurol*ogy. 63(4), 535-538. doi:10.1002/ana.21344.

Gregory, J.M., Barros, T.P., Meehan, S., Dobson, C.M., Luheshi, L.M. (2012). The Aggregation and Neurotoxicity of TDP-43 and Its ALS-Associated 25 kDa Fragment Are Differentially Affected by Molecular Chaperones in Drosophila. *PLoS ONE*. 7(2), e31899. doi:10.1371/journal.pone.0031899.

Kabashi, E., Valdmanis, P.N., Dion, P., Spiegelman, D., McConkey, B.J., Vande Velde, C., … Rouleau, G.A. (2008). TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nature Genetics*. 40(5), 572-574.

Korábečný, J., Spilovská, K., Benek, O., Musílek, K., Soukup, O., Kuča, K. (2012). Tacrine and its derivatives in the therapy of Alzheimer's disease. *Ceska a Slovenska farmacie.*61(5), 210-221.

Kwiatkowski Jr., T.J., Bosco, D.A., LeClerc, A.L., Tamrazian, E., Vanderburg, C.R., Russ, C., … Brown Jr., R.H. (2009). Mutations in the FUS/TLS Gene on Chromosome 16 Cause Familial Amyotrophic Lateral Sclerosis. *Science*. 323(5918), 1205-1208. doi:10.1126/science.1166066.

Lagier-Tourenne, C., & Cleveland, D.W. (2009). Rethinking ALS: the FUS about TDP-43. *Cell*. 136(6), 1001-1004. doi:10.1016/j.cell.2009.03.006

Lanson Jr., N.A., Maltare, A., King, H., Smith, R., Kim, J.H., Taylor, J.P., … Pandey, U.B. (2011). A Drosophila model of FUS-related neurodegeneration reveals genetic interaction between FUS and TDP-43. *Human Molecular Genetics*. 20(13), 2510-2523. doi:10.1093/hmg/ddr150.

Li, W.Z., Li, S.L., Zheng, H.Y., Zhang, S.P., Xue, L. (2012). A broad expression profile of the GMR-GAL4 driver in Drosophila melanogaster. *Genetics Molecular Research*. 11(3), 1997-2002.

Li, Y., Ray, P., Rao, E.J., Shi, C., Guo, W., Chen, X., … Wu, J.Y. (2010). A Drosophila model for TDP-43 proteinopathy. *Proceedings of the National Academy of Sciences of the United States of America*. 107(7), 3169-3174. doi:10.1073/pnas.0913602107

Maruyama, J., Yamagata, T., Ulfendahl, M., Bredberg, G., Altschuler, R., Miller, J.M. (2007). Effects of Antioxidants on Auditory Nerve Function and Survival in Deafened Guinea Pigs. N*eurobiology of Disease*. 25(2), 309-318. doi:10.1016/j.nbd.2006.09.012

McKee, A.C., Gavett, B.E., Stern, R.A., Nowinski, C.J., Cantu, R.C., Kowall, N.W., … Budson, A.E. (2010). TDP-43 Proteinopathy and Motor Neuron Disease in Chronic Traumatic Encephalopathy. *Journal of Neuropathology and Experimental Neurology*. 69(9), 918-929.

Murakami, T., Yang, S., Xie, L., Kawano, T., Fu, D., Mukai, A., … St George-Hyslop, P. (2011). ALS mutations in FUS cause neuronal dysfunction and death in Caenorhabditis elegans by a dominant gain-of-function mechanism. *Human Molecular Genetics*. 21(1), 1-9.

Neumann, M., Sampathu, D.M., Kwong, L.K., Truax, A.C., Micsenyi, M.C., Chou, T.C., … Lee, V. M.-Y. (2006). Ubiquitinated TDP-43 in Frontotemporal Lobar Degenerationand Amyotrophic Lateral Sclerosis. *Science*. 314, 130-133.

Rohn, T.Y. (2008). Caspase-Cleaved TAR DNA Binding Protein-43 is a Major Pathological Finding in Alzheimer's Disease. *Brain Research*. 1228, 189-198. doi:10.1016/j.brainres.2008.06.094.

Ross, C.A., & Poirier, M.A. (2004). Protein Aggregation and Neurodegenerative Disease. *Nature Medicine.* 10, S10-S17.

Rutherford, N.J., Zhang, Y.-J., Baker, M., Gass, J.M., Finch, N.A., Xu, Y.-F., … Rademakers, R. (2008). Novel Mutations in TARDBP (TDP-43) in Patients with Familial Amyotrophic Lateral Sclerosis. *PLoS Genetics*. 4(9), e1000193. doi:10.1371/journal.pgen.1000193.

Shiga, A., Ishihara, T., Miyashita, A., Kuwabara, M., Kato, T., Watanabe, N., … Onodera, O. (2012). Alteration of POLDIP3 Splicing Associated with Loss of Function of TDP-43 in Tissues Affected with ALS. *PLoS One*. 7(8), e43120. doi:10.1371/journal.pone.0043120.

Sieben, A., Van Langenhove, T., Engelborghs, S., Martin, J.-J., Boon, P., Cras, P., … Cruts, M. (2012). The genetics and neuropathology of frontotemporal lobar degeneration. Acta *Neuropathologica*. 124(3), 353-372. doi:10.1007/s00401-012-1029-x.

Smith, B.N., Newhouse, S., Shatunov, A., Vance, C., Topp, S., Johnson, L., … Shaw, C.E. (2012). The C9ORF72 expansion mutation is a common cause of ALS+/-FTD in Europe and has a single founder. *European Journal of Human Genetics*. doi:10.1038/ejhg.2012.98.

Southall, T.D., Elliott, D.A., Brand, A.H. (2008). The GAL4 System: A Versatile Toolkit for Gene Expression in Drosophila. *Cold Spring Harbor Protocols*. 79-95. doi:10.1101/pdb.top49

Sproviero, W., La Bella, V., Mazzei, R., Valentino, P., Rodolico, C., Simone, I.L., … Conforti, F.L. (2012). FUS mutations in sporadic amyotrophic lateral sclerosis: clinical and genetic analysis. *Neurobiology of Aging*. 33(4), 837.e1-5.

Sreedharan, J., Blair, I.P., Tripathi, V.B., Hu, X., Vance, C., Rogelj, B., … Shaw, C.E. (2008). TDP-43 Mutations in Familial and Sporadic Amyotrophic Lateral Sclerosis. *Science*. 319(5870), 1668-1672. doi:10.1126/science.1154584.

Suzuki, N., Kato, S., Kato, M., Warita, H., Mizuno, H., Kato, M., … Aoki, M. (2012). FUS/TLS-Immunoreactive Neuronal and Glial Cell Inclusions Increase With Disease Duration in Familial Amyotrophic Lateral Sclerosis With an R521C FUS/TLS Mutation. *Journal of Neuropathology and Experimental Neurology*. 71(9), 779-788.

Tsaytler, P., Harding, H.P., Ron, D., Bertolotti, A. (2011). Selective Inhibition of a Regulatory Subunit of Protein Phosphatase 1 Restores Proteostasis. S*cien*ce. 332(6025), 91-94. doi:10.1126/science.1201396

Ugras, S.E., & Shorter, J. (2012). RNA-Binding Proteins in Amyotrophic Lateral Sclerosis and Neurodegeneration. *Neurology Research International*. 2012, 432780. doi:10.1155/2012/432780.

Van Deerlin, V.M., Leverenz, J.B., Bekris, L.M., Bird, T.D., Yuan, W., Elman, L.B., … Yu, C.E. (2008). TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis. *Lancet Neurology*. 7(5), 409-416.

Vance, C., Rogelj, B., Hortobágyi, T., De Vos, K.J., Nishimura, A.L., Sreedharan, J., … Shaw, C.E. (2009). Mutations in FUS, an RNA Processing Protein, Cause Familial Amyotrophic Lateral Sclerosis Type 6. *Science*. 323(5918), 1208-1211. doi:10.1126/science.1165942.

Wang, J.-W., Brent, J.R., Tomlinson, A., Shneider, N.A., McCabe, B.D. (2011). The ALS-associated proteins FUS and TDP-43 function together to affect Drosophila locomotion and life span. *The Journal of Clinical Investiga*tion. 121(10), 4118-4126. doi:10.1172/JCI57883

Williams, K.L., Durnall, J.C., Thoeng, A.D., Warraich, S.T., Nicholson, G.A., Blair, I.P. (2009). A novel TARDBP mutation in an Australian amyotrophic lateral sclerosis kindred. *Journal of Neurology, Neurosurgery, and Psychiatry*.80(11), 1286-1288.

Xia, R., Liu, Y., Yang, L., Gal, J., Zhu, H., Jia, J. (2012). Motor neuron apoptosis and neuromuscular junction perturbation are prominent features in a Drosophila model of Fus-mediated ALS. *Molecular Neurodegeneration*. 7, 10. doi:10.1186/1750-1326-7-10.

You, Z., Savitz, S.I., Yang, J., Degterev, A., Yuan, J., Cuny, G.D., ... Whalen, M.J. (2008). Necrostatin-1 Reduces Histopathology and Improves Functional Outcome After Controlled Cortical Impact In Mice. *Journal of Cerebral Blood Flow and Metabolism*. 28(9), 1564-1573. doi:10.1038/jcbfm.2008.44

Acknowledgements

We would like to thank Dr. Jane Wu, the Charles Louis Mix Professor of Neurology and our research advisor at Feinberg School of Medicine for assisting us with project planning, providing the equipment, providing guidance throughout investigation, and reviewing our drafts of our research paper. We would also like to thank her for her advice for future work experiences. In addition, we would like to thank Mengxue Yang, a pre-doctoral student and our mentor for instructuing us in laboratory methods, helping us attain background information, assisting us set up experiment, and providing guidance throughout investigation. We would like to acknowledge the help of Xiaoping Chen, the Research Associate because she was always there to assist us with general laboratory tasks. Finally, we would like to thank Dr. Judith Scheppler and the SIR Staff for giving us the opportunity to conduct research at Feinberg School of Medicine.

Appendix

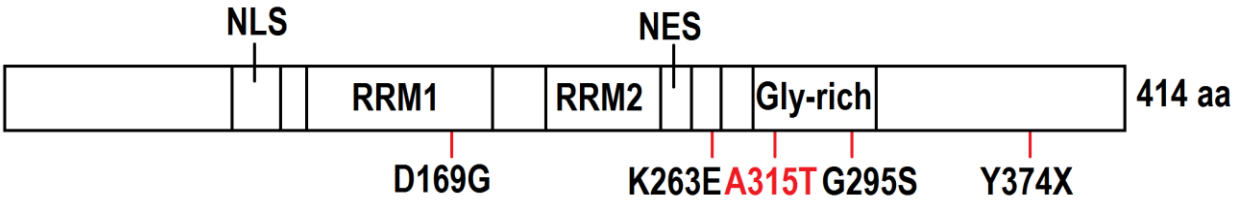

Figure 1. The gene map of TDP-43 gene.

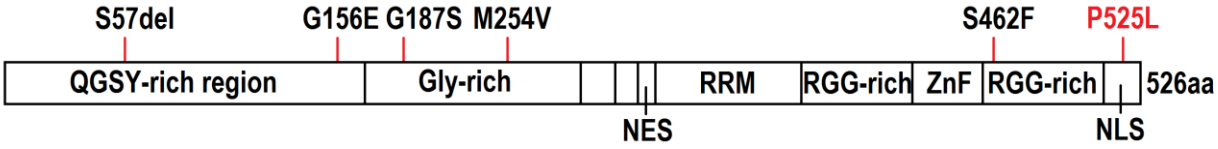

Figure 2. The gene map of FUS gene.

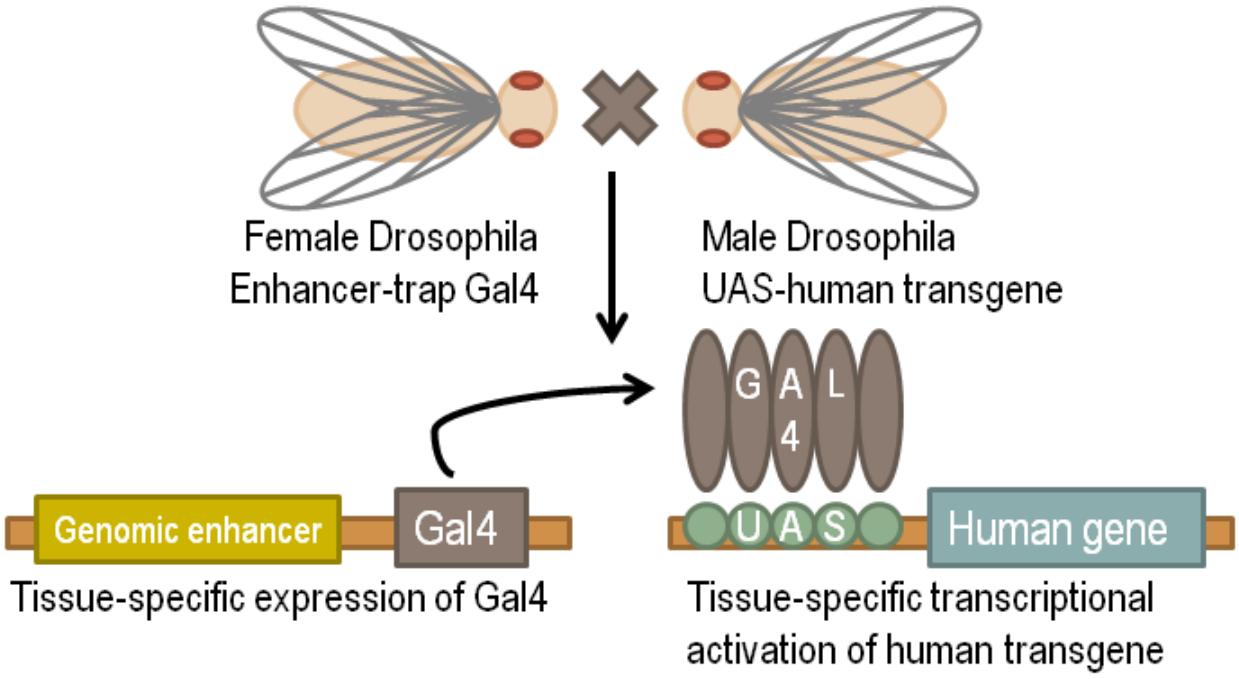

Figure 3. The Gal-4 System.

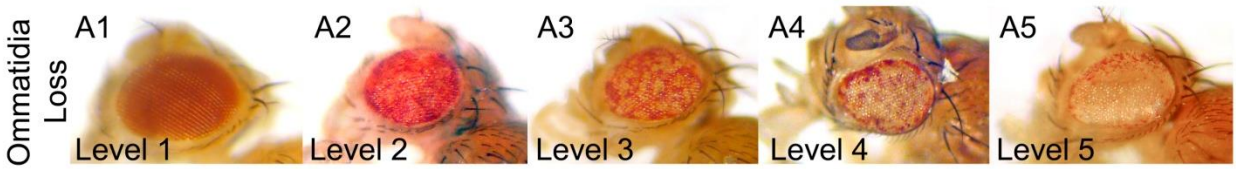

Figure 4. Eye Severity Criteria for *Drosophila* flies expressing TDP-43 and FUS in the eye.

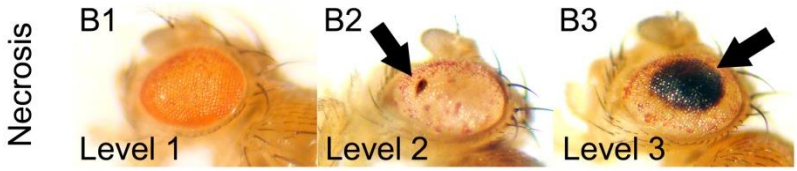

Figure 5. Eye Severity Criteria for *Drosophila* flies expressing TDP-43 and FUS in the eye.

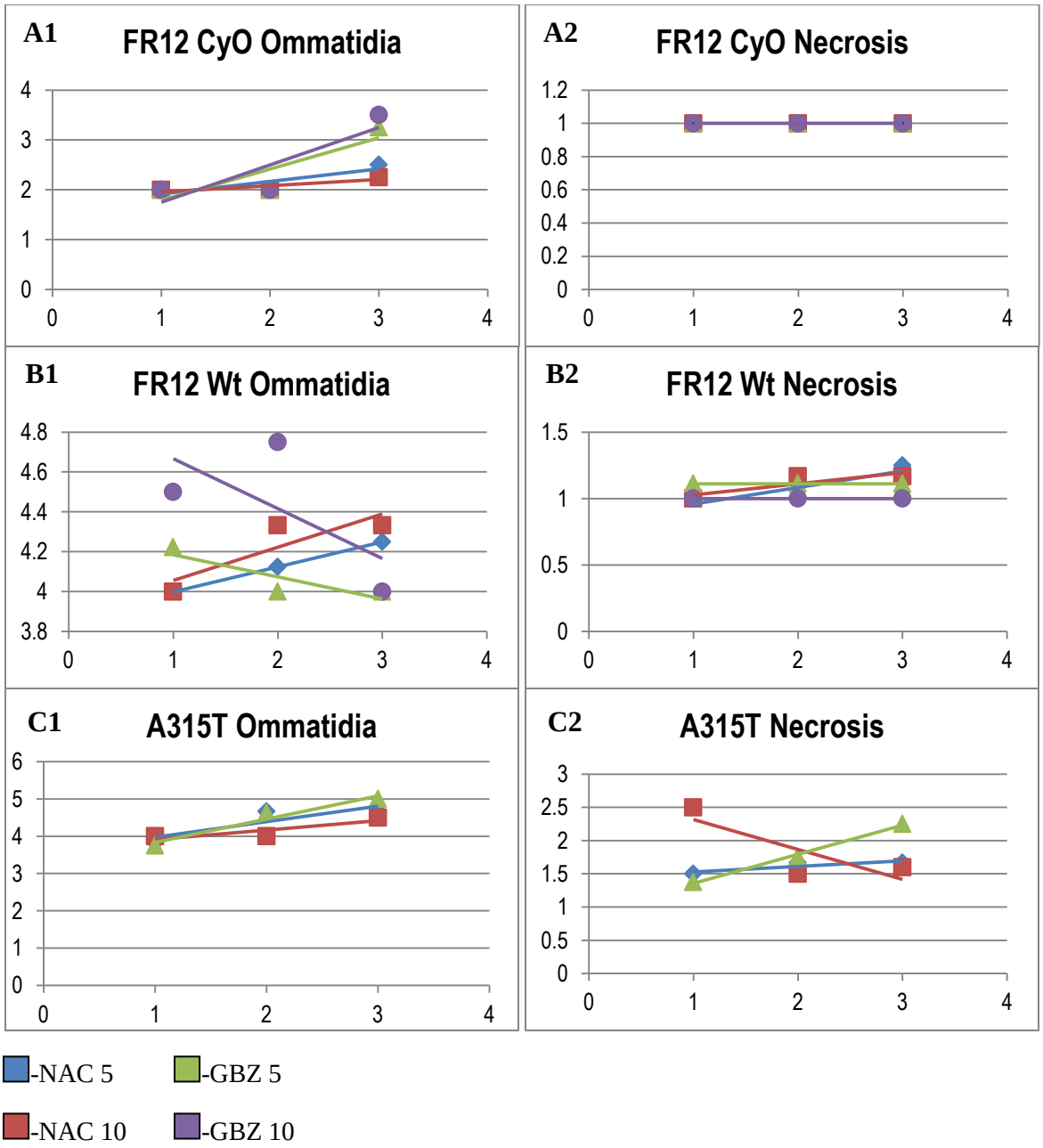

Figure 6. Average eye phenotype severity of Drosophila as it changes over time with a linear trendline. The x-axis represents time in terms of weeks and the y-axis represents the eye severity rank in the certain criterion.