AN INVESTIGATION OF THE INSULIN PATHWAY AND TDP-43 BASED AMYOTROPHIC LATERAL SCLEROSIS

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Abstract: The discovery of novel pathways and therapies in disease pathology is especially compelling in fatal neurodegenerative diseases like Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's Disease. We have developed a Drosophila model for ALS based on the overexpression of five variants of humanTDP-43, an RNA-binding protein that has been linked to ALS and other neurodegenerative diseases. In our fly model we can reproduce many of the ALS human pathologies including diminished locomotor function, and shortened life span. A primary screen of 1200 FDA approved compounds was performed, which led to the identification of compounds which rescue lethality, including six different antidiabetic drugs. These drugs fell into three different categories, namely sulfonylureas, biguanides, and thiazolidinediones. Follow-up validations studies have shown locomotor function defects caused by TDP-43 toxicity. These findings suggest that antidiabetic drugs may have therapeutic potential for ALS.

Introduction to Amyotrophic Lateral Sclerosis:

Amyotrophic Lateral Sclerosis, from now on abbreviated ALS, is a neurodegenerative disease that affects motor neurons. The disease is characterized by death of motor neurons of several areas including: the brain, spinal cord, motor cortex and other areas of the central nervous system. This pathology leads to loss of motor function, paralysis, in-coordination, and respiratory failure. Some forms of ALS have also been linked to forms of dementia and dystonia, leading to many questions on how the disease manifests. Often ALS is diagnosed through ruling out other motor and neurological diseases. Most patients diagnosed with this disease die within three to five years of diagnosis, usually due to respiratory failure. (Rowland and Shnider 2001, Jawaid et al 2010, Clark 2005, Boillee et al. 2006). This disease was first characterized in 1874 by French neurobiologist and practitioner, Jean-Martin Charcot. ALS was left a medical mystery for many decades, leaving the how and why this disease occurred left to be answered. The name came through common observation of the degeneration of both upper and lower degeneration of motor neurons. ALS was often times used to describe other disease such as progressive muscular atrophy, and bulbar palsy; all of which are now distinguishable from each other (Boillee et al., 2006, Clark 2005).

ALS consists of two major types: familial (fALS) and sporadic (sALS). Approximately ninety percent of ALS cases are sporadic, and the other ten percent are familial. There is also sex bias with this disease, which affects men more frequently than women; ratio of incident being 1.4:1.0. It is estimated that five to eight thousand US citizens are diagnosed with ALS each year, some researchers and physicians argue that this is a relatively low estimate (Clark 2005, Boillee et al, 2006).

Although there have been many discoveries and insight into the many causes and progression of ALS, still much is needed to be learned about this disease and what can be done for the patients and families affected by it.

TDP-43 based ALS Pathology:

Several loci have been linked to fALS. Mutations in superoxide dismutase, or SOD1, were among the first found in fALS. SOD1 is linked to around twenty percent of all fALS cases, or around two percent of all ALS diagnoses. Other loci have also been recently identified, one of them being TAR DNA-binding protein, or TDP-43 (Kabashi et al. 2010).

TAR DNA-binding protein (TDP-43) has a molecular weight of 43kD and contains 414 residues. TDP-43 has two RNA recognition motifs (RRM1 and RRM2) and a glycine rich domain in the C terminus. Mutations linked to ALS are mostly single amino acid substitutions and lie within the glycine rich C terminus with the exception of a single mutation found in the RNA recognition motif, RRM1. **Figure 1** below from C. Lagier-Tourenne and D. W. Cleveland (2009) shows where several mutations lie within the protein sequence. These mutations are thought to increase TDP-43's ability to phosphorylate and its targeting for degradation by the proteasome. This protein has been demonstrated to prepress transcription, regulate RNA splicing, mRNA shuttling, stability, and translation (Banks et al, 2008). TDP-43 is expressed in several tissues including the heart, liver, brain, kidney, and muscle (Neumann et al. 2006). TDP-43 is normally localized in the nucleus but is associates with cytoplasmic inclusions in disease samples. Similar inclusions have been found in *Drosophila* expressing the human form of TDP-43 when driven in the adult eye has shown these inclusions in retinal associated neurons (Estes et

al. 2011). Aggregates or stress granules have also been shown in the *C. elegans* model that formed in response to environmental stressors. (Lagier-Tourenne and Cleveland 2009).

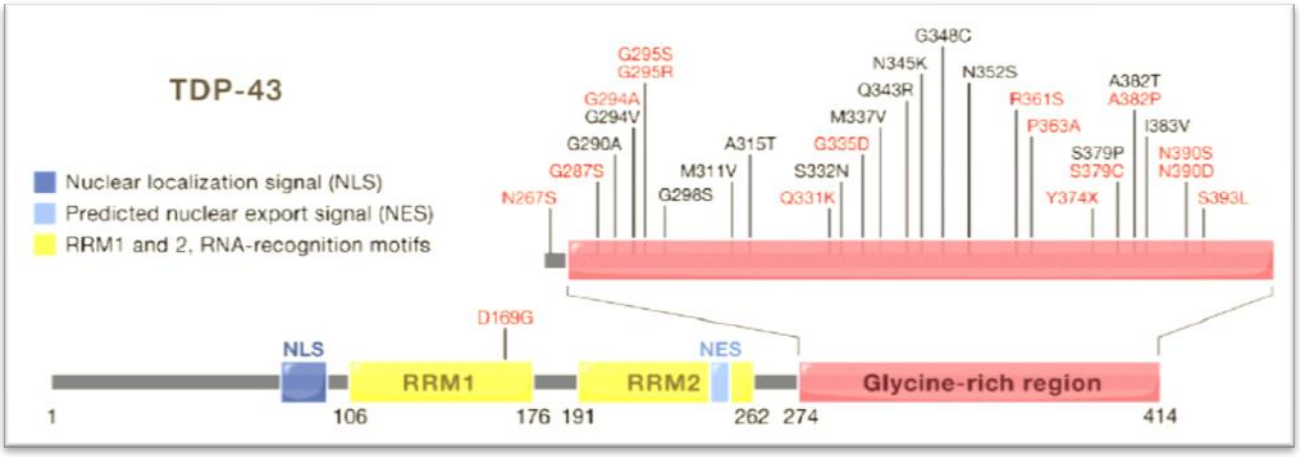

Figure 1: The various point mutations identified within TDP-43's RRM1, RRM2, and Glycine-rich C-terminus. Most of which are found within the C-terminus with the exception of D169G. The red colored mutations signify sporadic mutations, and the black mutations signify the familial cases. Figure from Lagier-Tourenne and Cleveland 2009

Modeling ALS in *Drosophila melanogaster*:

The ability to model human disease *in vivo* in model organisms can provide valuable insights into complex pathological processes. In order to study the specific disease related pathologies, it is crucial to be able to control for gene expression within that particular tissue of interest. Many animal models have systems that allow a particular protein or gene to be expressed in isolated tissue types, allowing for a spatial and temporal way to test various assays. The GAL-4 UAS system is a useful tool when driving expression of particular genes in different tissues in the *Drosophila* model. GAL4 is a yeast transcription activator, which regulars that transcription of a gene of interest via its binding site referred to as the Upstream Activating Sequences (UAS). GAL4 expressing fly lines casually referred to as drivers, can exert regulatory control in various tissues such as glial cells, muscle, retina, and motor neurons with high

specificity. With this system one can drive expression of fluorescently tagged proteins, which is useful in assays where visualizing regions or cells is crucial (Duffy 2002).

The Insulin Pathway and ALS:

The insulin pathway has been a target for many disease, pharmaceutical, and evolutionary investigations. Not only has the insulin pathway been linked to the disease diabetes mellitus but has other implications in ALS and neuronal function (Jawaid et al. 2010, Vaccaro et al. 2012, Bernardo and Minghetti 2008, and Kiaei 2008).

Peroxisome proliferator-activated receptor-γ (PPAR- γ) has a role in ALS and possibly glial cell functions. M. Kiaei (2008) showed that a small molecule PPAR- γ agonist (pioglitazone) had neuroprotective properties. This study compared findings of a SOD1 transgenic mouse, which when given the compound had a significant increase in survival, muscle strength, and even decreased weight loss. This agonist also reduced microglial activation and gliosis in the spinal cord. It is thought that this portion of the insulin pathway may have important implication in neuroinflammation, which is a commonly found in ALS and other neurodegenerative diseases. Bernardo and Minghetti (2008) also found that PPAR- γ agonist to have neuroprotective properties aiding in the slowing the progression of several neurodegenerative diseases including ALS. They found that PPAR- γ agonists (pioglitazone and rosiglitazone) aided in decreasing neuroinflammation and 2˚ neuronal damage, microglial activity, myelin loss, and neuropathic pain.

The insulin pathway has even been linked to TDP-43 related ALS specifically via the *C. elegans* orthologue TDP-1. Vaccaro et al. (2012) studied the insulin pathway's involvement in the regulation of stress signaling. They determined that a target within the pathway, DAF-16,

was involved in these regulatory processes of ALS pathology. Their results showed that by regulating DAF-16 transcriptional activity, the animals had decreased stress granules and extended lifespan. This process is thought to involve DAF-16 translocation to the nucleus where it activates numerous genes that are linked to survivorship and augmented stress resistance.

This connection to the insulin pathway and ALS is not limited to the model systems, but has also been shown in human patients. Jawaid et al. (2010) showed that patients with diabetes mellitus type 2 (DM) had later onset of ALS symptoms. This study compared 175 patients with ALS and DM to 2196 patients with just ALS. Their results showed that on average the age of onset for patients with ALS and DM was around 60.3 years compared to the non-diabetic group which age of onset was around 56.3 years, giving them a four year difference. They also tested the rate of progression and survival between these two groups finding that the trend continued where the diabetic group and a slower rate of progression and longer survival rates but the data failed to show statistical significance.

Materials and Methods:

Primary Screen Design:

Survivorship Assay: Male TDP-43 expressing transgenic flies was crossed with female D42 expressing flies. Three D42 females were crossed to two TDP-43 males. Specific variants include: wt2 1L , D169G3, G298S4, A315T9, and N345K3 . These flies are crossed and brooded on 1ml of food and drug mixture at either a 50 µM or 30 µM concentrations for seven days. After seven days the TDP-43 and D42 parents are removed. After several days the progeny are screened multiple times. Observations are recorded based on developmental progress, and validated through screening for Yellow Fluorescent Protein (YFP) expressed in the thoracic

ganglion. Below **Figure 2** is a schematic of the survivorship assay screening from food making to screening:

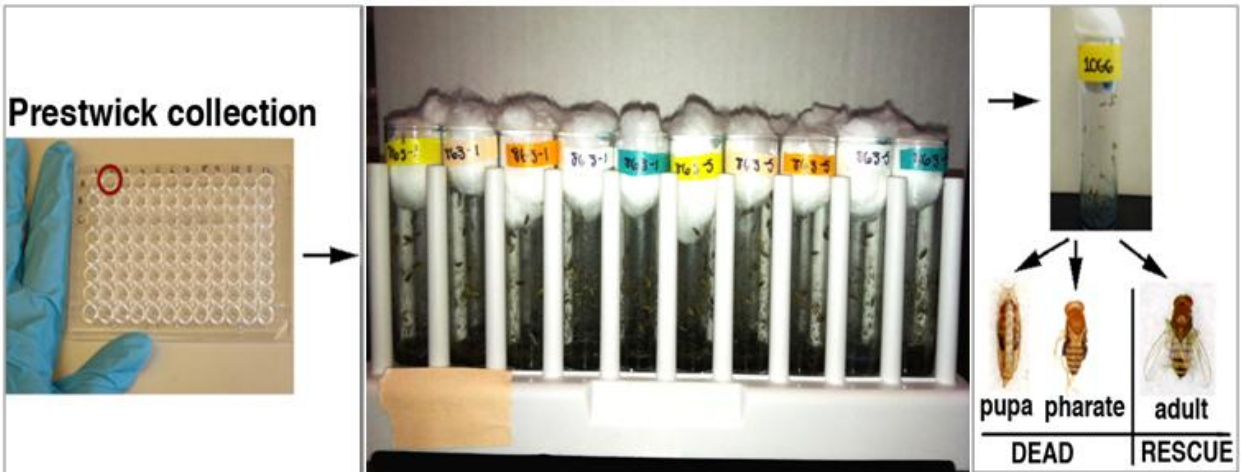

Figure 2: This shows the screen flowchart from using the drugs in the Prestwick collection to mixing them in food (see glass vials with 1ml of food and drug mixture) . Crosses are set up in these vials and screened for developmental stages and rescues.

Preparing the Drug and Food Mixture: Fifty milliliter bottles of stock food are created by a designated trained lab assistant. These bottles are melted down in a microwave until the food has formed a consistent liquid mixture. Once melted these bottles are placed in a hot water bath set at 55 degrees Celsius. The food is aliquoted into six milliliter amounts into glass vials, placed back into hot water bath, and capped. A glass vial containing the six milliliters of food is removed and dried. Drugs are added to a final concentration of 1, 5, 10, 25, or 50 µM to the melted food along with 100 µL of 1% bromophenol blue dye. 0.99-1.0 mL of the food and drug are pipetted into glass vials.

Antidiabetic Drug Survival Screen, and Larval Turning:

Antidiabetic Compound Survival Screen Design: Similar design to primary screen design but performed at 1, 5, 10, 25, or 50 µM concentrations, for a period of 25-30 days. At day 25 the pupae were counted and the number recorded.

Larval Turning Design: After the designated flies are removed from the drug and food mixture, as mentioned in the Antidiabetic Drug Larval Turning Food Design section, the larvae are raised until they reach late third instar larval stage of development, as denoted by vertically climbing up vial and leaving food. These larvae are removed and YFP confirmed on a grape juice agar plate. Once confirmed one larva at a time is flipped using a paint brush until it is ventral side up. The timer is started at this point and the larva is timed until it has returned to dorsal side up and has made one forward progression movement. Thirty larvae per genotype are tested.

Transgenics: Human TDP-43 YFP constructs were obtained from Aaron Gitler (Standford University) and cloned into the pUAST vector. After sequence verification plasmids were sent to Genetics Services for germline transformation. Approximately ten transformants per construct were screed and balanced in the laboratory.

Statistic analysis: The student's T-Test was performed using Microsoft Excel to determine statistical significance.

Results:

Intervention of small molecules helps to rescue lethality caused by human TDP-43 overexpression in Drosophila:

When raised at 25 degrees Celsius, progeny of the GAL-4 D42 x UAS TDP-43 cross do not survive past the larval or pupae stage. For this particular cross the GAL-4 D42 driver is

driving expression of TDP-43 in the motor neurons of these flies. Five different variants of TDP-43 were used, including wildtype (wt), three fALS linked mutations (G298S, A315T, and N345K), and one sALS mutation (D169G). Several classes of compounds were found to rescue lethality in the primary screen on more than one occasion. These compounds are classified based off the nomenclature provided in the Prestwick Library. These classes were picked based off the frequency of how many rescues were found to fall under each class. If as rescue was found more than once in a particular class it was considered in this group of data.

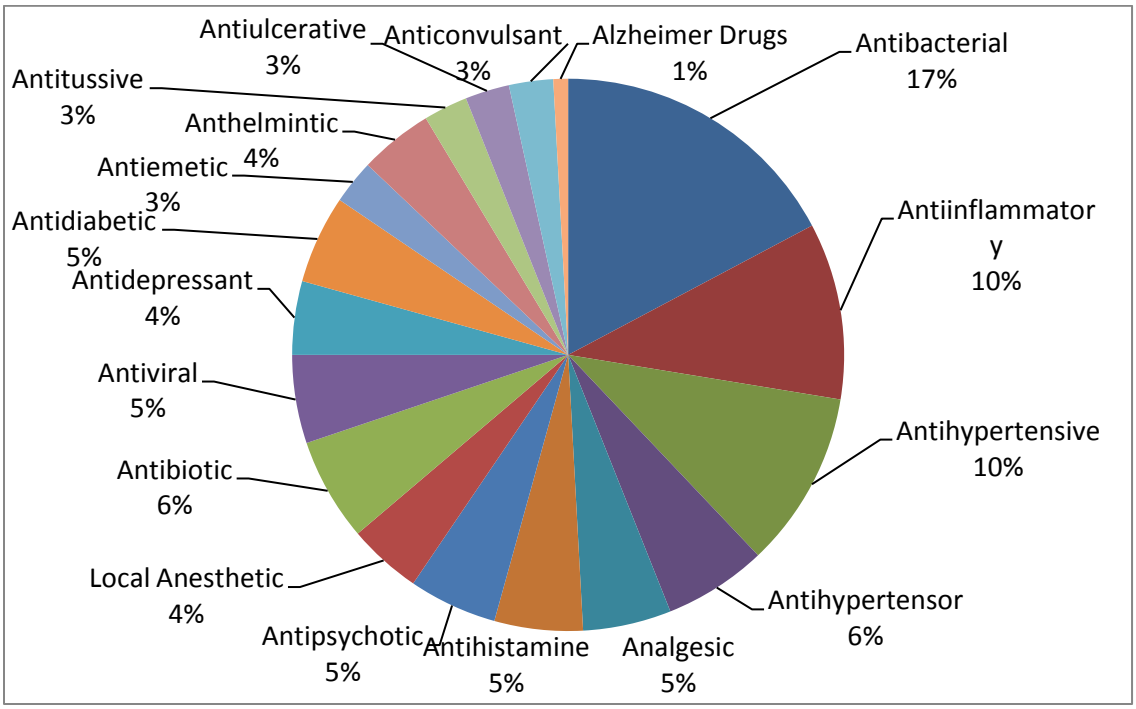

Figure 3: Various drug classes validated during the primary screen of the Prestwick Collection. This data set includes only those classes where more than one rescue was found.

There are also differences between the effects of various compounds on different human TDP-43 variants in regards to rescuing lethality. Of these positive compounds, a majority rescued wildtype (wt) (73.7%), followed by: G298S (15.7%), D169G (6.5%), A315T (2.8%), and N345K (1.4%). Only 5.5% of the drugs rescued more than one TDP-43 variant.

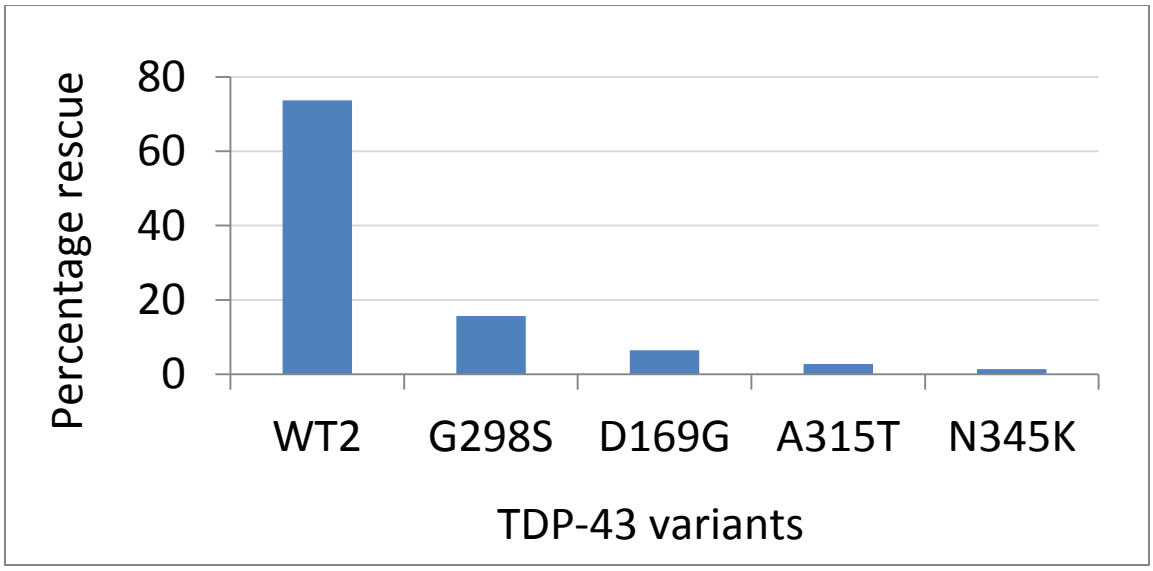

Figure 4: Percentage of TDP-43 variants rescued when crossed to D42 during the primary screen of the 1,200 compound Prestwick collection.

It is important to note the differences between the amounts of compounds rescued in a particular class compared to the number of total compounds of that class in the Prestwick Library. **Figure 5** shows that six antidiabetic drugs tested positive in our primary screen and that there were only fourteen available compounds in the library to test. The other classes of drugs within the library were not equally represented; some having only two compounds listed and other having more than one hundred compounds in one class.

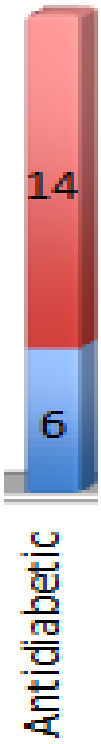

Figure 5: The number of antidiabetic drugs screened to be positive in a particular class versus the number of the total drugs in that class. Red: the total number drugs in the drug class. Blue: number of drugs validated in that particular class.

Survivorship Assay: The use of Pioglitazone, Metformin, and Gliquidone helps to rescue lethality in overexpressing human TDP-43 Drosophila:

After the extensive primary screen, it was evident that several classes had the ability to rescue TDP-43 lethality. However, it was the antidiabetic compounds seemed most promising based off the fact six of the fourteen compounds had validated in the primary screen, and all the promising results found between the insulin pathway and ALS. It worked out nicely that of the six drugs they were evenly distributed amongst three classes of antidiabetic compounds: sulfonylureas (gliquidone), biguanides (metformin), and thiazolidinediones (pioglitazone). **Figure 6** below shows where each of the classes, and the particular compounds chosen for the next set of assays, may possibly interact with components of the insulin pathway. One drug out

of each class was chosen to begin more extensive survival assays on based off their ability in the preliminary to rescue multiple TDP-43 variants.

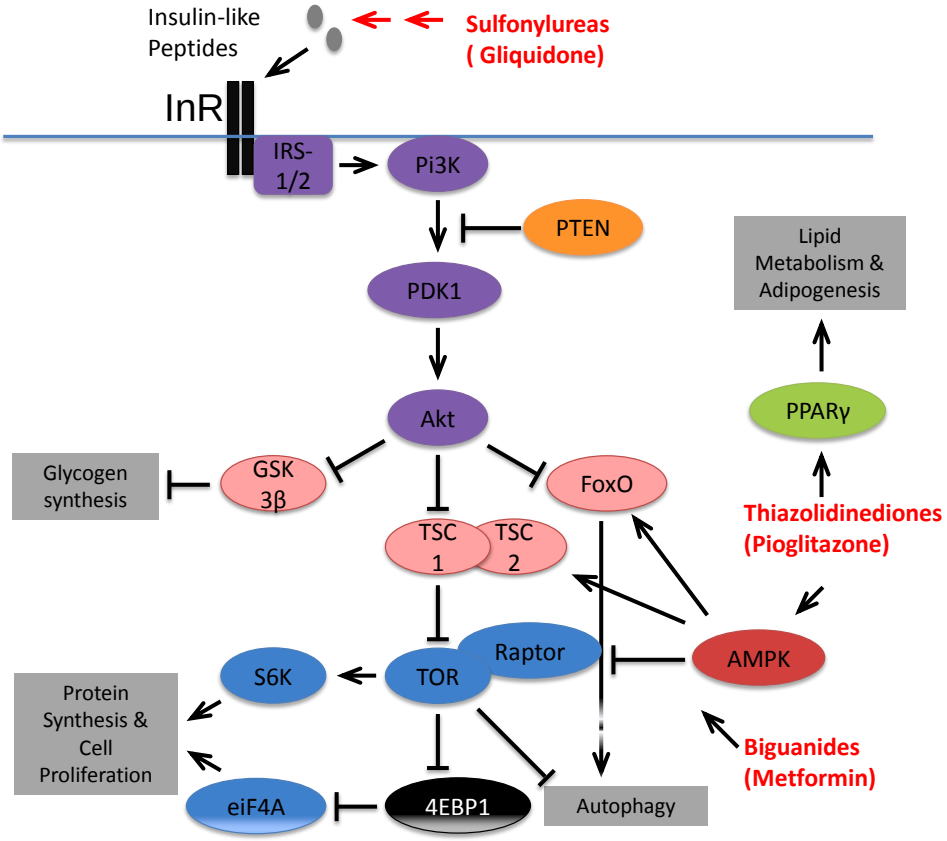

Figure 6: A simplified schematic of the insulin pathway flow chart and the possible targets for the different compounds. *Figure: courtesy of Andres Morera, graduate student, University of Arizona.*

The following data are the results of the continued survivorship assays done with Pioglitazone, Metformin, and Gliquidone. These were done over a series of twenty five to thirty days in triplicate in the five different concentrations of compounds and the control substance (DMSO) tested on the five different TDP-43 variants. Survivors were counted as normal straight winged flies that expressed YFP in their thoracic ganglion. After day twenty five the pupae were counted to assess the amount of survivors that hatched versus the amount of pupae that

developed. This assay was not done only to test for the rescuing effect of each of the compounds but also to see if there are differences between the concentrations.

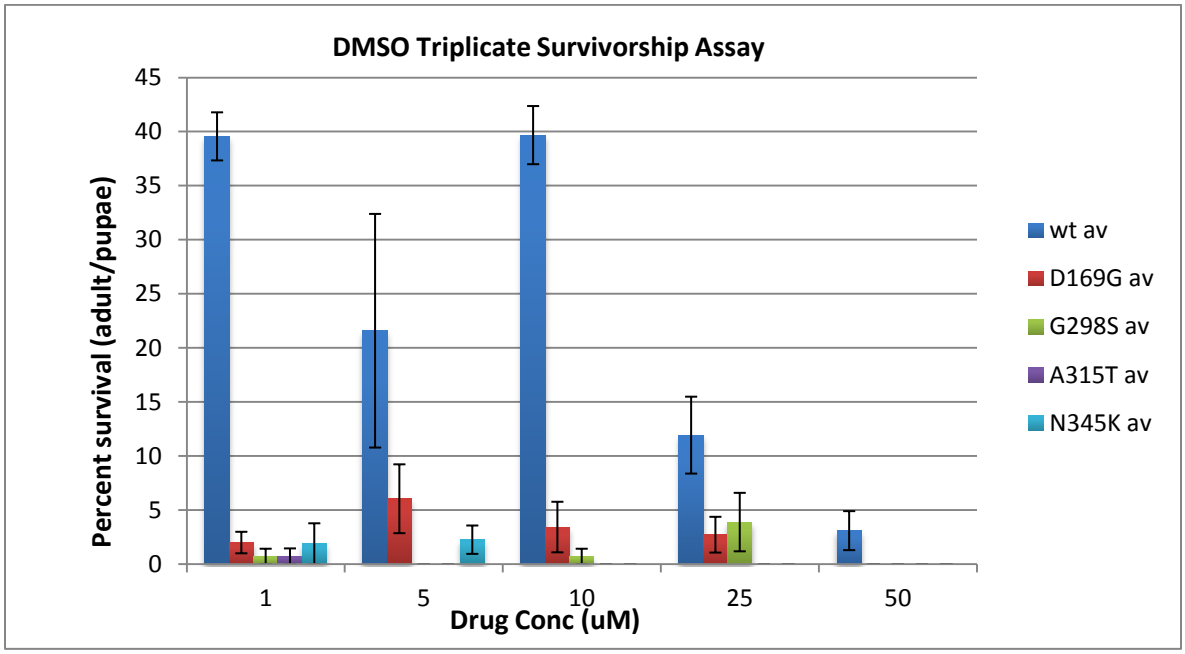

Figure 7: Results from the DMSO survivorship Assay. This is comparing the percent survival (adult survivals vs the number of pupae) against the DMSO concentrations.

Larval Turning Assay: Locomotor assays used to determine dose dependence of Antidiabetic compounds in overexpressing human TDP-43 Drosophila.

Due to the variability from the survivorship assays from the previous section, locomotor assays such as the larval turning test was used to help determine a dose response curve of the different concentrations of the three compounds. This test was only done on three variants: wt2, D169G, and G298S. Estes et al. (2011) showed that adult and larva TDP-43 expressing flies had significant locomotor dysfunction. By using a similar assay we compared the three compounds against the control for all three variants.

Significant results (p-value of 0.05 or less) for concentration 1 µM for D42 x hTDP-43 crosses include:

	wt2,h	D169G,h	G298S,h
Pioglitazone	1µM	ns at this concentration	1µM
Metformin	1µM	ns at this concentration	ns at this concentration
Gliquidone	1µM	ns at this concentration	1µM

Table 1: The significant larval turning results for a concentration of 1 µM for: Piolitazone, metformin, and gliquidone with the three TDP-43 variants. "h" stands for High expressor line

Discussion:

Antidiabetic compounds show rescue of lethality and improved locomotor function:

As mentioned previously, the insulin pathway has been shown to have significant effects on decreasing neuroinflammation and may possibly delay the onset of the disease in humans. Several studies have tested compounds targeting somewhere in this pathway and their results showed that these compounds have neuroprotective properties (Jawaid et al. 2010, Vaccaro et al. 2012, Bernardo and Minghetti 2008, and Kiaei 2008). Both Bernardo and Minghetti (2008) and Kiaei (2008) tested the compound a PPAR-γ agonist, pioglitazone, in their studies and had positive results with decreasing ALS pathology.

One of our compounds involved in these assays was the same compound tested by several of the studies mentioned previously. Pioglitazone showed to rescue lethality and improve locomotor coordination in wt2 and G298S. This result may be due to the neuroprotective properties that PPAR-γ agonists have within an organism. Although, there are not many studies to compare the other classes of compounds and their potential for being neuroprotective, some studies such as Jawaid et al (2010) showed a connection between patients with diabetes mellitus

had a delayed onset of ALS. The study does not say specifically if the patients were taking any antidiabetic compounds or what classes they were in so it is not known for sure if all classes of antidiabetic compounds are neuroprotective.

Survivorship assay failed to show a clear dose response curve or ideal concentration but did rescue multiple TDP-43 variants:

When looking at a compound's effectiveness it is important to find the range at which the drug is most effective. This is usually called a dose response curve where the ideal concentration of a given compound falls between a lower and upper limit. Along with the compound being tested in this manner it also usually tested pairwise with a control substance (Tice et al. 2000). Many of the adverse effects often seen with pharmaceutical prescribed compounds often occur at the upper end of this curve (MacDonald and Robertson 2009).

Our results from the survivorship assays are inconclusive with showing either an ideal concentration or a general trend with the concentrations of the different compounds. **Figure 5** shows just the results from the control compound, and it is evident that there is no clear dose response curve. Almost all of the variants fail to show the ideal bell shaped curve which would be necessary to identify the upper and lower limits of each compound. However, several of the compounds at certain concentrations were able to rescue all five TDP-43 variants; these results were not originally seen in the primary screen of the drugs which was performed at a relatively high concentration.

Larval Turning results showed ideal concentrations for each of the three compounds tested:

As previously mentioned locomotor dysfunction is a common phenotype in ALS human patients. Estes et al. (2011) showed that both wildtype and mutant forms had slower larval

turning times and adult climbing times than non TDP-43 expressing flies. There was also significant dysfunction in larval turning with their high expressing A315T mutant. Ash and colleagues (2010) showed similar locomotor phenotypes in *C. elegans*.

Our previous survivorship assays failed to show us a clear dose response curve, even after several trails that tried to lower the variability between the triplicate trials. Since larval turning is an effective assay in showing differences between TDP-43 expressing flies and control flies, we chose to use this assay as another way to test for a dose response curve (Estes et al. 2011). **Table 1** shows significant larval turning time differences of the compounds at a concentration of 1 µM for each of the compounds and each of the three variants. Wt2 was found to be effective in increasing larval locomotion at 1µM for all three compounds. G298S also showed significant rescuing results at 1 µM for pioglitazone and gliquidone. The only variant that did not significantly show differences between all three compounds and DMSO at this concentration was D169G.

Although there was not a clear lower limit for the dose response curve for these compounds there seems to be a general trend of lower concentrations are the most effective in rescuing this phenotype. Future tests are planned to test even lower concentrations of these compounds to see if the dose response lower limit can be reached.

Variability in the different variants of TDP-43:

Figure 4 showed that nearly 74% of all rescues in the primary screen were with wt2. This trend continued into the survivorship assays with the three antidiabetic compounds where once again wt2 had greater numbers of survivors. One explanation for this phenomenon is that over-expression of human wildtype TDP-43 has been shown in several studies to also have toxic

effects; these effects can vary depending on the tests performed. Also that wildtype TDP-43 can have more severe neuronal loss and neural muscular junction (NMJ) morphology issues when compared to the mutant forms (Lagier-Tourenne et al. 2010, Estes et al. 2011).

When looking at locomotor dysfunction Estes and colleagues (2011) showed that it was mutant A315T that had a more severe phenotype when compared to wildtype. Other models, such as *c. elegans,* have also shown locomotor dysfunction with higher levels of TDP-43 expression (Ash et al. 2010). Zebra fish and rats with mutant forms of TDP-43 have also expressed signs of neurotoxicity (Huang et al 2012, Kabashi et al. 2010).

Although not all forms of the TDP-43 variants from the primary screen have been tested in all aspects of this study, we can see from the larval turning data that each of the different variants have different results. Wt2 showed different results compared to the TDP-43 variants for the different concentrations for the different compounds. These results may likely show that each of the different TDP-43 variants are involved with different molecular mechanisms for each of the different compounds.

Future Directions:

Future directions for this project includes extending the dose dependence curve to see the extent of how even lower concentrations affect locomotor function and rescuing of lethality. Survival curves to test the life span of the flies raised on the antidiabetic drugs versus the control are currently underway. The rest of the five original variants are also being tested on the compounds in the larval turning assays. Other assays to be considered is the timing in which these drugs are necessary, whether it is a developmental effect or not. Genetic assays may also be performed on flies with insulin pathway mutations or deletions to see where along the insulin

pathway these drugs are actually working and if the compounds have any effect on their phenotype. Finally, several anatomical assays are underway. Active zone analysis of the neuromuscular junction are currently being imaged but will be analyzed later by other members on the project. Also thoracic ganglion dissections are in the works.

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