HIGH SCHOOL EDITION
Journal of Student Research
Volume 12 Issue 3 (2023)

Roscovitine's Effect on *D. melanogaster* with TDP-43 Nuclear Loss Amyotrophic Lateral Sclerosis

Mira Ramachandran[1], Satvika Aruva[1] and Jessica Eliason[1#]

[1]Academies of Loudoun
[#]Advisor

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a neurological disease that leads to motor neuron death, causing muscle atrophy and paralysis. The majority of ALS patients die from respiratory failure within 2–5 years. By 2040, the incidence of ALS is predicted to increase worldwide by 70%. ALS has no cure. TDP-43 protein dysfunction is present in ~97% of ALS patients. Past ALS research focused on TDP-43 aggregation in the cytoplasm of neuronal cells; however, loss of TDP-43 from the nucleus is now considered the main contributor to neurodegeneration. *Drosophila* larvae with dTDP-43 nuclear loss exhibit locomotion deficits and reduced levels of *cacophony*, a neuronal calcium channel required for neurotransmitter release. When *cacophony* was restored in dTDP-43 nuclear loss larvae, locomotion was rescued. Roscovitine is a drug that increases calcium influx in neuronal calcium channels, essentially performing the same function as increased *cacophony*. The purpose and novelty of this research are to determine if a roscovitine supplement can improve the locomotion of a TDP-43 nuclear loss ALS model of *Drosophila melanogaster*. The larval locomotion assay was used to validate the ALS symptom of muscle weakness. The movements of larvae on an agar plate were recorded. Using ImageJ, the displacements and speeds of the larvae were determined. Results indicate that ALS larvae fed roscovitine performed significantly better on the locomotion assay than ALS larvae fed normal food (p-value < 0.0001). This research provides insight into the role of neuronal calcium channels in TDP-43 nuclear loss and calcium channel agonists' potential in treating ALS.

1 | Introduction

1.1 Significance of Amyotrophic Lateral Sclerosis (ALS)

Amyotrophic lateral sclerosis (ALS), also known as "Lou Gehrig's disease," is a fatal disease that affects as many as 30,000 people in the United States, with over 5,000 new cases being discovered each year in the country (Talbott, 2016). ALS is characterized by progressive motor neuron deterioration. Motor neurons extend from the central nervous system to the spinal cord and are responsible for transmitting impulses to control muscle movements (Brotman et al., 2022). Early symptoms of ALS include muscle twitches, muscle cramps, muscle stiffness, and muscle weakness. ALS is progressive, meaning the symptoms get worse over time. Eventually all voluntary muscles are affected, and patients lose their ability to eat, speak, and move (Wijesekera & Leigh, 2009).

The US Food and Drug Administration (FDA) has so far approved two drugs to treat ALS: riluzole and edaravone. Riluzole is believed to reduce motor neuron damage by decreasing glutamate levels. Glutamate is the most abundant neurotransmitter released by nerve cells, and research indicates that excessive glutamate may cause motor neuron death. Clinical trials show that riluzole only prolongs survival by 2-3 months. Edaravone targets oxidative stress, which is reported to be another potential cause of motor neuron death. Unfortunately, edaravone has only slowed the decline of daily functioning in a small group of patients with early-stage ALS (Miller and Appel, 2017).

Therefore, current treatments are ineffective. Since the exact mechanisms behind ALS motor neuron death are unknown, more research on the pathology of ALS is needed for the development of an effective treatment.

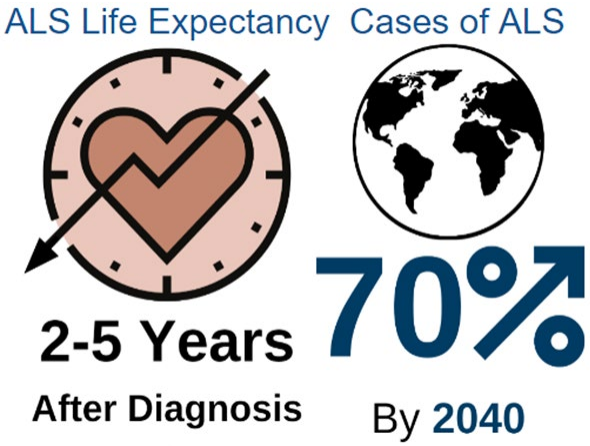


Figure 1. Current Life Expectancy and Future Projection of the Incidence of Amyotrophic Lateral Sclerosis.

1.2 TAR DNA-Binding Protein 43 (TDP-43) Pathology in ALS

Over 50 mutations in the TAR DNA-binding protein gene (TARDBP) have been linked to familial and sporadic cases of ALS (Gitcho et al., 2008). The TARDBP gene provides instructions for making the TDP-43 protein, which is ubiquitously expressed in all cell types but primarily localized to the nucleus. TDP-43 is a highly conserved protein, meaning it has remained relatively unchanged in evolutionary biology. It is an essential DNA-binding protein that regulates transcription, which is the initial step in producing proteins from genes (Tank et al., 2018). TDP-43 is also an RNA-binding protein that helps regulate messenger RNA (mRNA) homeostasis and splicing (Liu et al., 2019).

Approximately 97% of ALS patients, regardless of the mechanisms of disease onset, present dysfunction of the TDP-43 protein in post-mortem tissue. TDP-43 dysfunction is a central mechanism in the pathogenesis of ALS. There are two TDP-43 hypotheses that are believed to be contributing to neurodegeneration in ALS: TDP-43 cytoplasmic aggregation and TDP-43 nuclear loss. TDP-43 hyperphosphorylated aggregation has been observed in the cytoplasm of primarily neurons in ALS patients, which is considered a hallmark of the condition. Considerable attention has been given to TDP-43 cytoplasmic inclusions, as it is believed that they acquire toxic properties independent of the normal function of TDP-43, leading to the ALS pathology. However, critics argue, and new evidence suggests, that TDP-43 aggregation is merely a byproduct of neurodegeneration (Suk & Rousseaux, 2020). One study found that modeling TDP-43 aggregation in cells was correlated to cell death; however, when the aggregation models were treated with TDP-43 inhibiting synthetic peptides, cell death was not reduced or prevented. Thus, TDP-43 aggregation may not be the causative factor for cytotoxicity in ALS (Liu et al., 2013). By contrast, the hypothesis of TDP-43 nuclear loss states that lower levels of TDP-43 in the nucleus (where it is primarily localized) of neurons promote neurodegeneration. TDP-43 aggregation and nuclear loss are simultaneously present in ALS, but research suggests nuclear loss occurs earlier in the disease process. TDP-43 aggregation is a downstream consequence of nuclear loss. Therefore, therapeutically targeting the aggregation step may be too late to have a substantial impact on the disease. Targeting TDP-43 nuclear loss may have broader downstream effects on disease mechanisms and prevent or slow down the changes that ultimately lead to aggregation (Suk & Rousseaux, 2020). The loss of TDP-43 from the nucleus is a significant feature of ALS that requires further investigation.

1.3 TDP-43 in Drosophila melanogaster

Our research aims to investigate the role of TDP-43 nuclear loss in ALS using *Drosophila melanogaster* (fruit flies) as the model organism. The *Drosophila* ortholog of human TDP-43 is TBPH. TBPH is expressed throughout development and has been detected in neurons. Like TDP-43 in humans, TBPH is primarily localized in the nucleus. (Layalle et al., 2021). Suppression of TBPH in the nucleus of neurons was found to result in impaired larval locomotion and a reduced number of axonal branches and synaptic boutons, which are also hallmarks of ALS in humans. Axon branching is critical to ensuring that electrical impulses within a neuron are changed into chemical messages in the form of neurotransmitters, and synaptic boutons store the neurotransmitters to communicate with other neurons. *Drosophila* locomotion as well as the number of axonal branches and synaptic boutons were rescued by the expression of human TDP-43 in neurons, indicating TDP-43 is necessary to maintain these functions. This study of TBPH suppression demonstrates that TDP-43 function is necessary to regulate locomotive behaviors, and TBPH suppression can cause the neurological issues observed in ALS in the absence of aggregation formation. The TBPH ortholog and the classic ALS hallmarks of *Drosophila melanogaster* support the utility of the fly to study TDP-43 dysfunction in ALS.

There are a few ways to model TDP-43 nuclear loss in *Drosophila melanogaster*. One of the methods is the GAL4/UAS system and RNA interference (RNAi)-induced gene knockdown. The GAL4 gene originates from yeast and encodes the GAL4 transcriptional activator protein. The gene is inserted into the *Drosophila* genome and placed under the control of an enhancer, which controls GAL4 expression. When the enhancer is active, GAL4 is expressed. The enhancer is tissue-specific, meaning it only activates the transcription of GAL4 in specific types of cells in the fly. The Upstream Activation Sequence (UAS) is a segment of DNA that binds with GAL4 to promote the transcription of a reporter gene, which is a gene of choice. The GAL4 (driver line) and UAS (reporter line) are in separate flies. When the two lines of flies are crossed, the generation of flies created contains both the GAL4 and UAS genes. In the cells where GAL4 is expressed, the GAL4 protein will bind to the UAS, initiating the expression of the reporter gene (Osterwalder et al., 2001). RNAi is a system that inhibits the synthesis of proteins through the degradation of mRNA with the help of several different enzymes. TPBH can be suppressed in *Drosophila* neurons using the GAL4/UAS system and RNAi. The UAS-TBPH RNAi line can be crossed with the Elav-GAL4 (pan-neuronal) line to knockdown TBPH in *Drosophila* neurons. The progeny of this cross has been previously found to display impaired larval locomotion and a reduced lifespan, which makes the RNAi *Drosophila* model an appropriate method to study TDP-43 nuclear loss (Layalle et al., 2021).

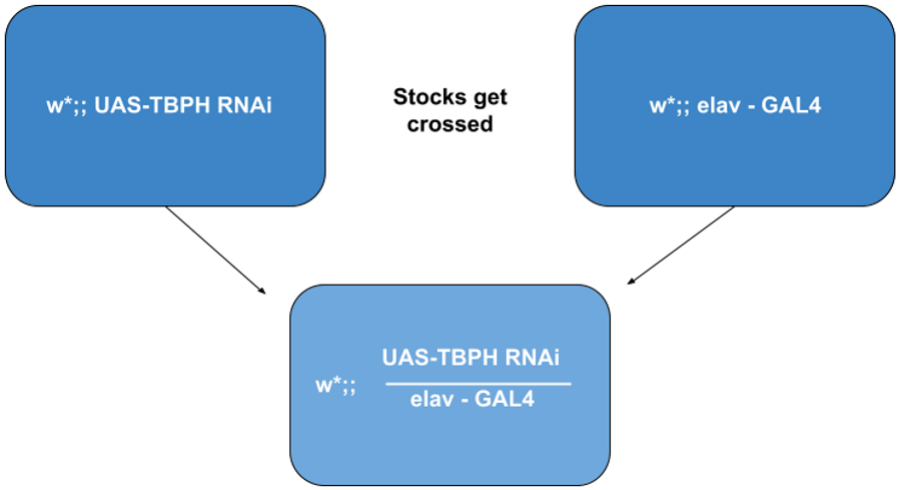


Figure 2. Cross for TDP-43 Nuclear Loss Flies. The dark blue boxes represent the names of the stocks used. The blue boxes represent the 4 chromosome structure of *Drosophila* stocks that are crossed. The light blue box is the final TDP-43 nuclear loss genotype of ALS.

1.4 Cacophony in Drosophila melanogaster with TDP-43 Nuclear Loss

Due to TDP-43's role in RNA processing and splicing, loss of TDP-43 results in abundant splicing changes and global gene expression changes (Liu et al., 2019). The central nervous system of third-instar larvae was examined, and about 1000 genes were identified to show splicing changes in TBPH nuclear loss larvae compared to wild-type larvae. The gene encoding a CaV2 calcium channel, named *cacophony*, showed the highest rate of splicing changes, rendering *cacophony* less functional. CaV2 calcium channels, also called the N and P/Q voltage-gated calcium channels (VGCC), are channels found in the brain. VGCCs are involved in vesicle release at synaptic terminals (Chang et al., 2014). Synaptic terminals are found at the ends of axons, where an action potential, or an electrical impulse, is converted into a chemical signal (Südhof, 2012). Neurotransmitters carry chemical signals in synaptic vesicles, which are essential for neuronal communication. Calcium influx at the CaV2 channels triggers vesicle release. The *cacophony* protein is localized at the neuromuscular junction (NMJ), which is the connection between the terminal end of a motor neuron and a muscle. *Cacophony* is responsible for the majority of neuronal calcium currents and is also required for neurotransmitter release (Chang et al., 2014).

Loss of TBPH in *Drosophila* causes reduced levels of *cacophony*. Restoring *cacophony* expression either pan-neuronally or selectively in motor neurons rescues the locomotion defects in larvae caused by loss of TBPH. *Cacophony* was found to be a direct target for TBPH loss, which indicates that a Ca_V2 calcium channel agonist, or an agent that increases calcium influx, can be used to treat TBPH nuclear loss and prevent motor neuron death in ALS (Chang et al., 2014).

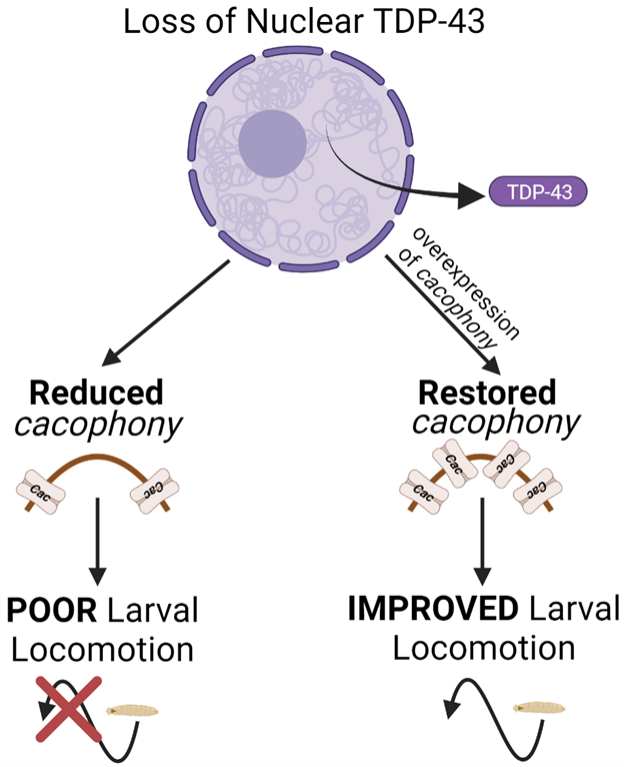


Figure 3. TDP-43 Nuclear Loss Pathology *Drosophila melanogaster.*

1.5 Roscovitine as a Potential Treatment for TDP-43 Nuclear Loss ALS

Roscovitine is a drug widely used to inhibit cyclin-dependent kinase 5 (CDK5), a protein expressed in the brain that plays a key role in brain development and neuronal cytoskeleton structure. Abnormal CDK5 activity is implicated in a variety of neurological disorders. Roscovitine also has an effect on calcium currents in neurons independent of its

CDK5 behavior. Using voltage-clamp recordings, a method used to measure ion currents through cell membranes, roscovitine was found to modify the voltage-dependent gating of calcium channels in the neurons of mice. Roscovitine prolonged the open state of the P/Q-type calcium channels, resulting in increased calcium influx (Yan et al., 2002). To reiterate, *cacophony* in *Drosophila* is a P/Q-type calcium channel. Therefore, roscovitine could potentially treat TDP-43 nuclear loss ALS in *Drosophila*.

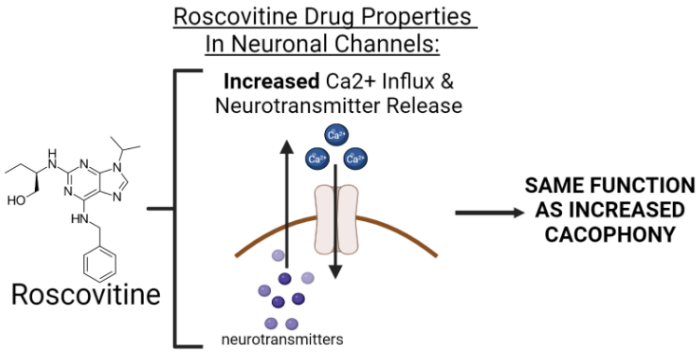


Figure 4. Roscovitine Drug: Calcium Channel Agonist.

1.6 Purpose, Novelty, Variables, Controls, and Hypothesis

The purpose of our research is to determine if a roscovitine treatment can improve the locomotion of a TDP-43 nuclear loss Amyotrophic lateral sclerosis model of *Drosophila melanogaster*. There is no research regarding pharmacological treatments for TDP-43 nuclear loss ALS, even though it is now considered the primary driver of neurodegeneration. Testing a roscovitine treatment on this particular, insufficiently researched model of ALS is an aspect of the novelty of this project.

This research has two independent variables: the supplementation of roscovitine and the TDP-43 nuclear loss model of ALS. Two diets will be fed to the larvae: a standard diet and a roscovitine-enriched diet. The roscovitine toxicity was determined through concentration testing since roscovitine's effect on flies is untested.

Given that restoring levels of *cacophony* in *Drosophila* larvae with loss of TBPH rescues their locomotion defects and roscovitine's calcium agonist activity, it was hypothesized that roscovitine would improve the locomotion of larvae with the TDP-43 nuclear loss model of ALS.

There are three control groups in this experiment: larvae with ALS and without the roscovitine supplement; larvae without ALS and with the roscovitine supplement; and larvae without ALS and without the roscovitine supplement. Larvae of either the UAS-TBPH RNAi or elav-GAL4 genotype were used as control flies without ALS because gene expression is silent in the uncrossed stocks.

The dependent variable is the locomotion behavior of the *Drosophila* larvae. ALS has been validated by larval locomotion assays in the past, as a primary symptom of ALS is muscle weakness. Third-instar larvae will be used and will be placed on an apple juice agar plate. The crawled paths of the larvae will be video recorded and then uploaded to a software called ImageJ. The ImageJ wrMtrck plugin will determine the displacement of the larval paths (mm) and the speed of the larvae (mm/second) (Brooks et al., 2017). The loss of TBPH protein in *Drosophila* results in severe deficits in larval locomotion and is late pupal lethal. Therefore, the larval locomotion assay is appropriate for this experiment (Chang et al., 2014).

2 | Methods

2.1 Materials

The fly stocks used to create the TDP-43 nuclear loss model were UAS-TBPH RNAi and elav-GAL4. Both fly stocks were obtained from the Bloomington *Drosophila* Stock Center at Indiana University, with stock numbers #29517 and #8760, respectively. Roscovitine (10 mg) was obtained from AdooQ Bioscience with catalog number A10806. The apple juice for making the apple juice agar plate was Mott's 100% Original Apple Juice and was purchased from our local grocery store. All other equipment mentioned in the following methods needed for fly maintenance and disposal, fly food preparation, CO_2 anesthetization and fly crosses, locomotion assay, and ImageJ analyses was obtained from the Academies of Loudoun. The ImageJ software was downloaded from http://imagej.nih.gov/ij/. The wrMTrck plugin was downloaded from http://www.phage.dk/plugins/wrmtrck.html.

2.2 Culture and Maintain Flies

Flies were kept in plastic vials with cotton plugs and reared at room temperature (22°C) to ensure a 2-week life cycle. When the fly stocks were expanded, flies were transferred to new vials every 4 days. When fly stocks were maintained, flies were transferred to new vials every 3 weeks. Dry food vials were moisturized with distilled water. Flies were disposed of by freezing them in a -20°C freezer for 1 hour.

2.3 Normal (Untreated) Fly Food Preparation

Using a scoopula, an electronic scale, and weigh boats, 6.75 g of yeast, 3.90 g of soy flour, 28.50 g of yellow cornmeal, and 2.25 g of agar were measured. A graduated cylinder was used to measure 30 mL of light corn syrup and 390 mL of distilled water. The wet and dry ingredients were mixed in a 500-mL beaker using a glass stirring rod. Then, the ingredients were microwaved in 30-second increments and stirred between the increments to prevent overflow. The ingredients were microwaved until the mixture came to a boil. After microwaving, the beaker was covered with cheesecloth to prevent contamination. The mixture was allowed to cool until there was no more steam coming out. Then, 1.88 mL of propionic acid was added to prevent mold. Empty vials were filled with approximately 10 mL of food and plugged. Throughout experimentation, vials were monitored for mold and mites.

2.4 Fly Cross to Model TDP-43 Nuclear Loss

Virgin adult female flies of either the UAS-TBPH RNAi or elav-GAL4 genotype were combined in a vial with a male fly from the other genotype. The larvae of the cross exhibit the TDP-43 nuclear loss genotype and the ALS phenotype.

To obtain virgin females of a particular genotype, the flies were anesthetized using CO_2. The nozzle of the CO_2 gun was inserted into the fly vial. The flies were only exposed to the gas for a few seconds. Then, the anesthetized flies were placed on a CO_2 sorting pad under a light microscope. The microscope was used to identify the virgin females. The males of the other genotype were anesthetized and identified as well. The selected females and males were placed into an empty food vial to create the cross.

Virgin females were collected on the day of or shortly after their emergence. The virgin collection vials were created approximately 14 days prior to cross-creation to maximize the number of virgins found. The age of the male flies of the other genotype did not matter. The males and females used to create the cross were transferred to new vials and reused once to collect more ALS progeny. After one reuse, new crosses were created.

2.5 Roscovitine Concentration Testing and Treatment Food Preparation

10 mg of roscovitine was dissolved in 14.104 mL of dimethyl sulfoxide (DMSO) to create a 2 mM stock solution. Then, the stock solution was diluted to make fly vials with 10 mL of 1 µM and 10-100 µM food. The 10-100 µM range was created in increments of 10 µM. Varying amounts of roscovitine DMSO stock solution were mixed into the normal fly food (see Section 2.3) after the food was microwaved and cooled to make roscovitine food of different concentrations. Two vials were made for each of the 11 concentrations. 10 adult flies of either the UAS or GAL4 genotype were placed in each concentration testing vial. After 7 days, the percentage of surviving flies in each vial was calculated. 85% of the adult flies at the 20 µM roscovitine concentration survived. Additionally, abundant larvae were visible in the 20 µM concentration vials. Concentrations higher than 20 µM reared close to no larvae. Thus, a 20 µM roscovitine concentration was used to make the experimental fly food.

To prepare the 20 µM roscovitine experimental food, 2.307 mL of the 2mM roscovitine stock solution was mixed with 228.393 mL of normal food (see Section 2.3). Several vials were created. The treatment vials were stored in a 4°C refrigerator.

2.6 Larval Locomotor Assay

To make the apple juice agar plate, combine 185 mL of apple juice, 192 mL of distilled water, and 7.0 g of agar powder in a 500-mL beaker. These ingredients were mixed using a stirring rod. The mixture was placed in the microwave and taken out after 2 minutes. The mixture was mixed again. Then, the mixture was placed back into the microwave in 30-second increments and mixed between the increments until it reached a boil. After microwaving, the mixture was allowed to cool. Once the beaker glass was warm to the touch, 4.0 mL of acetic acid, 4.2 mL of ethyl alcohol, and 10 drops of bromophenol blue dye were added to the mixture. The acetic acid and ethyl alcohol help preserve the agar plate. The blue dye ensures the background is dark enough for contrast enhancement, so ImageJ can track the larvae. Then, a large weigh boat (140 mm x 140 mm) was obtained. The apple juice mixture was poured into the weigh boat. Once the gel was completely hardened, the plate was plastic-wrapped and stored in a 4°C refrigerator. The locomotion assay was never conducted on the same day the plate was made, as the plate may have been too soft (Brooks et al., 2017).

To extract larvae for the locomotor assay, small agar plates were created. The apple juice agar recipe was also used to make small agar plates. The agar solution was added to petri dishes, and the small plates were stored in the refrigerator. Then, the experimentation vial with larvae was obtained. The vial was created approximately 7 days before experimentation to allow for the extraction of plenty of third-instar larvae. A 20% sucrose solution was created by dissolving 3 g of sucrose into 15 mL of distilled water. The sucrose solution was poured into the experimentation vial, and the larvae were allowed to float to the top for 5-10 minutes. A plastic pipette was used to transfer the larvae to an empty petri dish. Then, distilled water was added to the petri dish to clean the larvae. From the petri dish, the larvae were transferred to a small agar plate using tweezers (Nichols et al., 2012).

To conduct the locomotion assay, the agar plate was placed under a shelf. An iPhone was placed on the shelf so that the camera protruded and captured the entire agar plate. With tweezers, the larvae were transferred from the small agar plate to the center of the large agar plate. ImageJ can track five larvae on the plate at a time (five larvae per trial). Once all five larvae were on the plate, a timer for 4 minutes and a video recording were started. At the end of the 4 minutes, the iPhone video was ended. The larvae were transferred to a kim wipe and disposed of. Plates were reused for experimentation. New plates were made every two weeks. It was very important not to accidentally puncture the plate with the tweezers throughout the experimentation, as the larvae can easily burrow themselves into the gel (Brooks et al., 2017).

2.7 Image J Analysis

The iPhone locomotion video was converted to a ".avi" file and uncompressed using **Virtual Dub**. The .avi video file was opened on the ImageJ software. The 'convert to grayscale' option was selected. The entirety of the agar plate was selected as the area of interest using the rectangular tool (Image>Crop). The video was inverted so the larvae appeared dark on a light background (Edit>Invert). Substacks of 1788 frames, corresponding to 29.8 frames/second, were created to analyze 60 seconds worth of video data (Image>Stacks>Tools>Make Substack). The subtract background function was used to remove smooth, continuous backgrounds (Process>Subtract background). The Kalman Stack Filter option was selected to remove potential light flickering during video creation (Plugins>stacks>Kalman Stack). The image threshold was adjusted to increase the contrast of pixels corresponding to larvae (Image>Adjust>Threshold; use default settings and deselect 'dark background') (Brooks et al., 2017). The wrMTrck plugin was run (Plugins>wrMTrck>wrMTrck), and settings were modified to the **optimal settings** for the tracking of the larvae, specifically analyzing path length, path trace, and raw data for movement parameters. The wrmTrck output assigns a number to each larva tracked, which corresponds to raw data displayed in a results table as well as a summary table displaying the average displacement in mm and speed in pixels/second for the five larvae (Brooks et al., 2017).

2.8 Safety Precautions

Closed-toe shoes were worn throughout the experimentation. When propionic acid, acetic acid, ethanol, bromophenol blue dye, and roscovitine were handled, gloves and goggles were worn, as these substances can cause serious eye and skin irritation. When hot substances were handled, hot hands were used.

3 | Results

3.1 ImageJ Data: Larval Displacements and Speeds

Table 1. Average Displacement[1] (mm) and Average Speed[2] (mm/sec) of the Larvae per Trial for Each Group

	No ALS Normal Food		**ALS Normal Food**		**No ALS Roscovitine Food**		**ALS Roscovitine Food**	
Trials	**D[1] (mm)**	**S[2] (mm/sec)**	**D (mm)**	**S (mm/sec)**	**D (mm)**	**S (mm/sec)**	**D (mm)**	**S (mm/sec)**
1	28.384	0.531	12.548	0.259	28.126	0.562	20.214	0.427
2	25.185	0.486	18.275	0.361	31.696	0.608	19.126	0.397
3	27.188	0.498	15.200	0.324	30.097	0.585	19.912	0.427
4	28.354	0.593	13.313	0.293	29.183	0.576	21.337	0.445
5	28.860	0.631	15.105	0.328	27.066	0.539	20.974	0.434
6	29.499	0.702	16.889	0.350	28.808	0.578	20.794	0.437
7	29.250	0.574	16.162	0.319	31.362	0.607	21.959	0.456
8	27.569	0.568	15.770	0.343	30.176	0.584	20.489	0.424
9	31.256	0.606	16.806	0.341	28.985	0.566	20.653	0.418
10	26.319	0.529	16.392	0.363	30.509	0.561	20.625	0.429
11	32.405	0.603	15.541	0.351	29.149	0.531	21.084	0.442
12	29.023	0.558	17.115	0.381	28.604	0.542	19.875	0.392
13	30.475	0.590	15.603	0.340	27.504	0.565	20.116	0.402
Means	**28.751**	**0.575**	**15.748**	**0.335**	**29.328**	**0.570**	**20.551**	**0.425**

3.2 Speed Sample Calculation

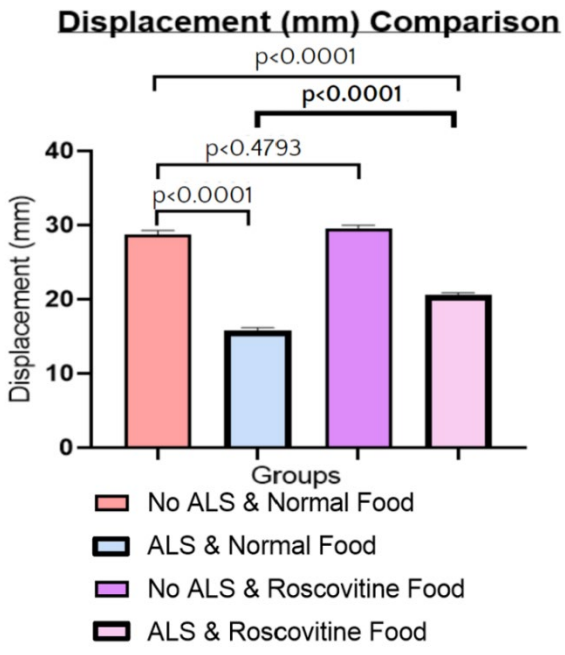
ImageJ records the speed of larvae in pixels per second. To convert it to mm/second, we needed to determine a mm/pixels conversion factor. To do this, we found the length of the agar plate on ImageJ in pixels (1052 pixels) using the straight line tool. Then, we found the length of the actual agar plate in mm (140 mm). Our conversion factor was 140 mm/1052 pixels.

Group: No ALS & Normal Food
Trial 1 Speed Calculations:

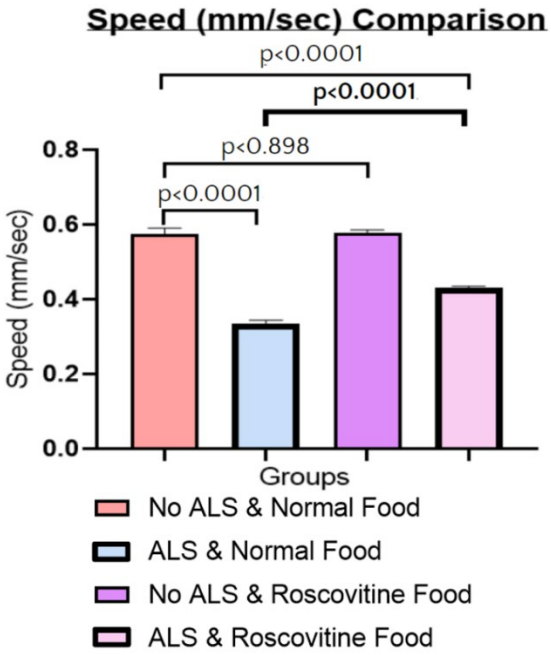
$$\frac{3.990\ pixels}{second} \cdot \frac{140\ mm}{1052\ pixels} = 0.531\ mm/second$$

3.3 Graphs with Mann-Whitney U Test p-value[1] Comparisons

Graph 1. Average Displacement (mm) for Each Group



Graph 2. Average Speed (mm/sec) for Each Group



[1]p-values in bold are considered significant for being <0.05.

The values in Table 1 were used for statistical analyses. All values were rounded to three decimal places, which is a measurement uncertainty. Additionally, the length in pixels of the agar plate slightly changed for each video. The speed calculations were processed twice, which is another measurement uncertainty.

3.4 Larval Path Comparison

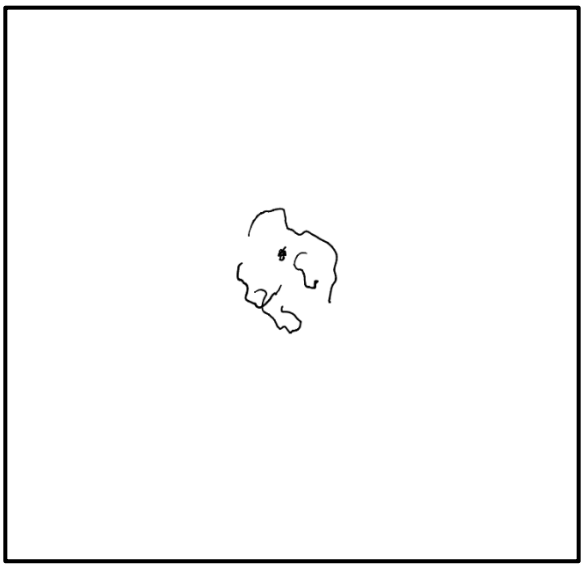


Figure 5. ALS Larvae and Normal Food Locomotion Path from ImageJ. ALS larvae fed normal food tend to make more turns. They do not travel far from their starting point.

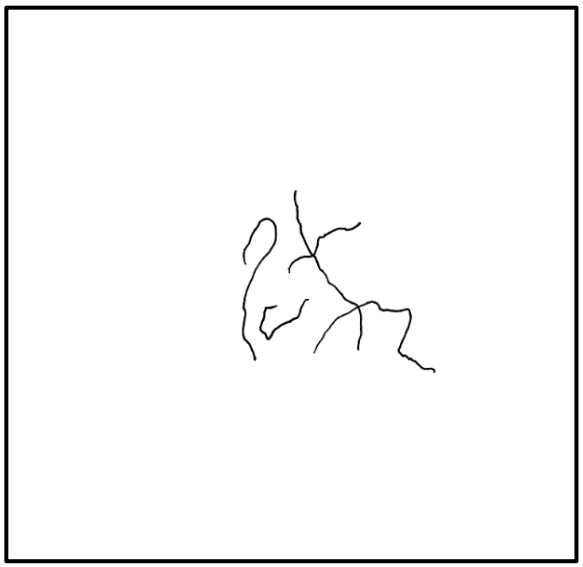


Figure 6. ALS Larvae and Roscovitine Food Locomotion Path from ImageJ. ALS larvae fed roscovitine food tend to make fewer turns. They are more coordinated and travel farther from their starting point.

3.5 Analysis of Results and Hypothesis

It was hypothesized that roscovitine could improve the locomotion of TDP-43 nuclear loss ALS larvae. Graphs 1 and 2 depict the p-values for the larval displacement and speed comparisons. ALS larvae fed roscovitine food performed significantly better on the locomotion assay than ALS larvae fed normal food regarding both displacement ($p<0.0001$) and speed ($p<0.0001$); our hypothesis is supported.

ALS larvae performed significantly worse on the locomotion assay than healthy larvae regarding both displacement ($p<0.0001$) and speed ($p<0.0001$), thus validating the TDP-43 nuclear loss ALS mechanism. Roscovitine did not have a significant effect on the locomotion of healthy larvae regarding both displacement ($p=0.431$) and speed ($p=0.7438$). Therefore, roscovitine alone does not have an impact on locomotion. Roscovitine's capacity to improve ALS larval locomotion can be attributed to its calcium channel agonist activity and ability to target TDP-43 nuclear loss, which are also illustrated in Figures 6 and 7. Lastly, healthy larvae fed normal food performed significantly better on the locomotion assay than ALS larvae fed roscovitine food regarding both displacement ($p<0.0001$) and speed ($p<0.0001$), indicating that roscovitine cannot make ALS larvae locomotion comparable to healthy larvae.

4 | Discussion

4.1 Potential Errors

During experimentation, there were some sources of potential error. Fruit fly larvae are sensitive to changes in temperature. While the temperature of the lab remained constant at 22℃, the agar plate was stored in the refrigerator at 4℃. Changes in temperature may have affected the speed of the larvae on the plate. In order to account for this, we took the agar plate out of the fridge during the larvae extraction process to allow the plate to acclimate to the lab

temperature before the locomotion assay. Nevertheless, it is difficult to ensure that the temperature of the plate is exactly for every trial. Slight variations would have occurred that affected our locomotion results.

Another possible source of error was the larvae's response to sucrose during the larval extraction process. In our initial trials, the larvae were directly transferred from the sucrose solution to the locomotion assay plate. We found that this drastically reduced locomotion as the larvae were dirty. Additionally, the larvae would be exposed to the sucrose solution for varying amounts of time. The longer they were exposed to sucrose, the slower they would move. We redid these trials and added the steps of rinsing the larvae with water and drying them on small agar plates. Also, all larvae were transferred to the small agar plates at once before experimentation to keep the amount of time each larva was exposed to the sucrose similar. While larval locomotion was restored through the addition of these steps, it is difficult to fully account for any confounding effects that may be caused by the sucrose solution.

4.2 Connections to Past Research

One study investigated the effect of GV-58 in a Lambert-Eaton myasthenic syndrome (LEMS) mouse model. LEMS is an autoimmune disorder affecting the NMJ. It is characterized by the reduction of functional P/Q-type calcium channels at motor neuron terminals. The reduction of functional VGCCs leads to a decrease in neuronal calcium influx, which decreases chemical neurotransmission, leading to neuromuscular weakness. GV-58 is a roscovitine analog that is adjusted to increase its efficacy as a calcium channel agonist and decrease its efficacy as a CDK antagonist. GV-58 was found to directly target P/Q-type calcium channels and partially restore the deficiency of neurotransmitter release (Tarr et al., 2013). Both TDP-43 dysfunction in ALS and LEMS are characterized by a decrease in calcium influx at neuronal calcium channels (CaV2), and the roscovitine analog is reported to be an effective calcium channel agonist in LEMS. This research is consistent with our findings: roscovitine is an appropriate agent for targeting disorders characterized by P/Q-type calcium channel dysfunction.

Another study discussed the correlation between ALS and L-type calcium channels, which are found in postsynaptic neurons. It is known that ALS patients often present with increased levels of glutamate. Glutamate is a neurotransmitter released by L-type calcium channels (Foran and Trotti, 2009). The increased activity of L-type channels is associated with motor neuron death; however, anti-glutamatergic ALS treatments targeting L-type channels were primarily ineffective in human trials (Miller and Appel, 2017). However, our results indicate that restoring activity in P/Q-type channels (found in pre-synaptic terminals) can restore locomotion in ALS. Our research emphasizes the necessity of studying the seemingly opposite implications of L and P/Q type calcium channels in ALS further.

5 | Conclusion

5.1 Limitations

A major limitation of this research is that roscovitine was developed for the purpose of inhibiting CDK 5. Due to cost restrictions, we were unable to buy P/Q-type calcium channel agonists with solely agonist activity. However, since the targeted TDP-43 nuclear loss mechanism is specifically characterized by reduced calcium channels and not abnormal CDK5 activity, our results can be attributed to roscovitine calcium channel agonist activity.

5.2 Future Work

Future work could study roscovitine's effect on the eclosion rate of TDP-43 nuclear loss larvae. Roscovitine may significantly impact lifespan and may allow nuclear loss larvae to reach the adult fly stage.

5.3 Impact

Roscovitine's effect on the displacement and speed of ALS larvae indicates improved muscular strength. Thus, our results suggest that roscovitine may slow the process of muscle atrophy, improving our currently limited understanding of TDP-43 nuclear loss and ALS. The calcium channel agonist properties of roscovitine can be harnessed to develop a novel ALS treatment.

6 | Acknowledgments

We would like to thank our research mentor, Dr. Jessica Eliason, for her guidance and support. We would also like to thank the Academies of Loudoun for providing the necessary equipment for experimentation.

7 | References

Brooks, D. S., Vishal, K., Kawakami, J., Bouyain, S., & Geisbrecht, E. R. (2016). Optimization of wrMTrck to monitor Drosophila larval locomotor activity. *Journal of Insect Physiology*, *93–94*, 11–17. https://doi.org/10.1016/j.jinsphys.2016.07.007

Brotman, R. G., Moreno-Escobar, M. C., Joseph, J., & Pawar, G. (2022). Amyotrophic lateral sclerosis. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK556151/

Chang, J.-C., Hazelett, D. J., Stewart, J. A., & Morton, D. B. (2014). Motor neuron expression of the voltage-gated calcium channel cacophony restores locomotion defects in a Drosophila, TDP-43 loss of function model of ALS. *Brain Research*, *1584*, 39–51. https://doi.org/10.1016/j.brainres.2013.11.019

Foran, E., & Trotti, D. (2009). Glutamate transporters and the excitotoxic path to motor neuron degeneration in amyotrophic lateral sclerosis. Antioxidants & Redox Signaling, 11(7), 1587–1602. https://doi.org/10.1089/ars.2009.2444

Gitcho, M. A., Baloh, R. H., Chakraverty, S., Mayo, K., Norton, J. B., Levitch, D., Hatanpaa, K. J., White, C. L., Bigio, E. H., Caselli, R., Baker, M., Al-Lozi, M. T., Morris, J. C., Pestronk, A., Rademakers, R., Goate, A. M., & Cairns, N. J. (2008). TDP-43 A315T mutation in familial motor neuron disease. *Annals of Neurology*, *63*(4), 535–538. https://doi.org/10.1002/ana.21344

Layalle, S., They, L., Ourghani, S., Raoul, C., & Soustelle, L. (2021). Amyotrophic lateral sclerosis genes in drosophila melanogaster. *International Journal of Molecular Sciences*, *22*(2), 904. https://doi.org/10.3390/ijms22020904

Liu, E. Y., Russ, J., Cali, C. P., Phan, J. M., Amlie-Wolf, A., & Lee, E. B. (2019). Loss of nuclear tdp-43 is associated with decondensation of line retrotransposons. Cell Reports, 27(5), 1409-1421.e6. https://doi.org/10.1016/j.celrep.2019.04.003

Liu, R., Yang, G., Nonaka, T., Arai, T., Jia, W., & Cynader, M. S. (2013). Reducing TDP-43 aggregation does not prevent its cytotoxicity. Acta Neuropathologica Communications, 1(1), 49. https://doi.org/10.1186/2051-5960-1-49

Miller, R. G., & Appel, S. H. (2017). Introduction to supplement: The current status of treatment for ALS. *Amyotrophic Lateral Sclerosis & Frontotemporal Degeneration*, *18*(sup1), 1–4. https://doi.org/10.1080/21678421.2017.1361447

Nichols, C. D., Becnel, J., & Pandey, U. B. (2012). Methods to assay drosophila behavior. *Journal of Visualized Experiments : JoVE*, *61*, 3795. https://doi.org/10.3791/3795

Osterwalder, T., Yoon, K. S., White, B. H., & Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. *Proceedings of the National Academy of Sciences*, *98*(22), 12596–12601. https://doi.org/10.1073/pnas.221303298

Südhof, T. C. (2012). Calcium control of neurotransmitter release. *Cold Spring Harbor Perspectives in Biology*, *4*(1), a011353. https://doi.org/10.1101/cshperspect.a011353

Suk, T. R., & Rousseaux, M. W. C. (2020). The role of TDP-43 mislocalization in amyotrophic lateral sclerosis. *Molecular Neurodegeneration*, *15*(1), 45. https://doi.org/10.1186/s13024-020-00397-1

Tank, E. M., Figueroa-Romero, C., Hinder, L. M., Bedi, K., Archbold, H. C., Li, X., Weskamp, K., Safren, N., Paez-Colasante, X., Pacut, C., Thumma, S., Paulsen, M. T., Guo, K., Hur, J., Ljungman, M., Feldman, E. L., & Barmada, S. J. (2018). Abnormal RNA stability in amyotrophic lateral sclerosis. *Nature Communications*, *9*(1), 2845. https://doi.org/10.1038/s41467-018-05049-z

Tarr, T. B., Malick, W., Liang, M., Valdomir, G., Frasso, M., Lacomis, D., Reddel, S. W., Garcia-Ocano, A., Wipf, P., & Meriney, S. D. (2013). Evaluation of a novel calcium channel agonist for therapeutic potential in Lambert-Eaton myasthenic syndrome. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *33*(25), 10559–10567. https://doi.org/10.1523/JNEUROSCI.4629-12.2013

Wijesekera, L. C., & Nigel Leigh, P. (2009). Amyotrophic lateral sclerosis. Orphanet Journal of Rare Diseases, 4(1), 3. https://doi.org/10.1186/1750-1172-4-3

Yan, Z., Chi, P., A. Bibb, J., A. Ryan, T., & Greengard, P. (2002). Roscovitine: A novel regulator of P/Q-type calcium channels and transmitter release in central neurons. *The Journal of Physiology*, *540*(3), 761–770. https://doi.org/10.1113/jphysiol.2001.013376