USING *C. ELEGANS* AS A MODEL SYSTEM TO DISCOVER ANTIEPILEPTIC DRUGS

by

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This thesis was prepared under the direction of the candidate's thesis advisor, Dr. James K. Wetterer, and has been approved by the members of her supervisory committee. It was submitted to the faculty of the Honors College and was accepted in partial fulfillment of the requirements for the degree of Bachelor of Arts in Liberal Arts and Sciences.

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Abstract

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Novel antiepileptic drugs (AEDs) are commonly tested using rats and mice as model systems. These animals, however, require a great deal of time, money, and lab space to maintain. In contrast, the nematode *Caenorhabditis elegans* (*C. elegans*) potentially can provide a faster, and less expensive model system for testing AEDs. C. elegans are simple to maintain and have a very short generation time, allowing high throughput assays to screen for new AEDs. For my thesis research, I used *C. elegans* to test FDA approved AEDs. My tests support the contention that *C. elegans* can be a useful model system for AED discovery.

To Stephanie

Table of Contents

Introduction

Seizures and Epilepsy

Seizures are a prevalent and damaging health problem in populations. It can negatively affect daily life for many people. A seizure is a loss of neuronal homeostasis while epilepsy is a disorder one has when they experience two or more seizures. In humans, 10% of people will experience a seizure and one out of twenty six in the population will develop epilepsy (Hesdorffer *et al.*). This can be significantly detrimental to daily life and can cause those suffering from seizures to be unable to complete with daily tasks. It can also interfere with complex tasks such as disrupting driving.

Seizures can be caused by a birth defect, electrolyte imbalance, drug and alcohol abuse or withdraw and channel malfunctions (Delanty, Vaughan and French, 1998). Some birth defects can include ion channels malfunctioning in the brain. Sodium channels historically play a role in seizures as these channels are caused to stay open.

Potassium channels have also been shown to play in a role in seizures as well (Sharon *et al.*, 1998). Potassium channels are a downstream target of Protein Kinase G (PKG), and by opening them, we hypothesize that there would be a protective effect. PKG has not previously been implicated in seizures studies.

Electroconvulsion is a way to cause seizures that has been shown in *D. melanogaster* larvae and is therefore implicated in epilepsy (Marley and Baines, 2011).

Two different genetic strains of *C. elegans* were used in this research, *N2* which is a wild type and *egl-4* which is a hypomorph that has reduced PKG (reduced protein levels) and therefore, reduced potassium channel activity downstream (Dawson-Scully *et al.*, 2010). The *N2* should be assumed to recover faster from an electroconvulsive seizure

than the *egl-4* hypomorph. These two genetic strains are shown in this research to be significantly different in time to recovery from the onset of an electroconvulsive shock and therefore seizure. Antiepileptic drugs (AEDs) are pharmacological aids that help to reduce the duration of seizures.

Retigabine is a recent FDA approved AED that targets potassium channels and is implicated in seizure studies. Preliminary research showing Retigabine successfully reducing seizure time encouraged the propagation of this research to further AEDs as it showed they have potassium channel homologs to the ones in rats and mice (Wei, Butler and Salkoff, 2005).

Model System

Seizure and epilepsy models currently exist in rats and mice, which therefore receive most of the funding for seizure research (White, 1997). These mammalian organisms have long life spans and long generation times, and therefore current novel drug testing in these animals takes a considerable amount of time and money to complete.

Caenorhabditis elegans (C. elegans) are small microscopic nematodes and were chosen as a model system for many reasons including their known genetics, ease of care and use. They can be stored in large quantities in a small space and are inexpensive to maintain. These animals also can easily be used for a high throughput assay due to their small size and quick generation time.

C. elegans go through several life stages, shown in Figure 1, including a stage called L4 which occurs when they are approximately 24 hours old. The L4 stage is denoted by a light semicircle appearing near the center of the body. For this research, all

worms that were used of all strains were pulled aside into their own plate at L4 stage and given another 24 hours to mature into one day old adults.

Two genetically different strains of *C. elegans* were used: *N2* and *egl-4* (*egl-4* (n478)IV strain MT1073) worms. The *N2* worms are a wildtype and have an average amount of PKG while the *egl-4* worms are a hypomorph loss-of-function strain that has less active PKG than N2 and could result in dysfunctional potassium channels causing seizure susceptibility. The *egl-4* worms express significantly less PKG than the average PKG animals, the *N2*s. These specific mutants were chosen as we have seen in preliminary trials that in *D. melanogaster*, a difference in varying amounts of PKG affects seizure susceptibility.

C. elegans are a useful model system as they are the answer to many of the issues that researchers face when using rats or mice including financial, genetic, time and space limitations. They are small and cost very little to purchase and maintain. Their connectomes are mapped and their 302 neurons are well known down to where they synapse. *C. elegans* are currently used as models for neurodegenerative diseases, and they have previously shown use in dealing with epilepsy (Bessa, Maciel, and Rodrigues, 2013). Simple model systems like this are very useful tools for studying advanced diseases such as Alzheimer's disease, Parkinson's disease, epilepsy and Autism spectrum disorders as "*C. elegans* displays complex behaviors such as learning and habit formation and even presents some degree of social interaction" (Bessa, Maciel, and Rodrigues, 2013). Many of the genetic mutations that cause these neurodegenerative diseases and neurological disorders in humans have homologs in *C. elegans*.

Previous research has been done with inducing seizures in *C. elegans (*Williams *et al.,* 2004*).* These seizures were caused by paralysis that is mechanistically similar to humans by using Aldicarb, induced by high temperatures, or susceptible mutant genetic backgrounds with proconvulsants such as pentylenetetrazole (Vashlishan *et al.*, 2008; Pandey *et al*., 2010; Williams *et al.*, 2004). Causing seizures in *C. elegans* is not new, however causing seizures using electroconvulsion is novel.

Antiepileptic Drug History

Most likely due to this long period of testing that must occur with mammalian test subjects, most of the current AEDs that have been approved by the Food and Drug Administration (FDA) are older like Phenobarbital and Phenytoin which were discovered to work with those suffering from epilepsy in 1912 and 1936 respectively (Baumann and Ryan, 1999) and are still in use today.

Preliminary studies were completed by Monica Risley, FAU PhD candidate, to determine the usefulness of AEDs through the use of Retigabine in the *C. elegans* electroconvulsive assay. *C. elegans* showing a significantly reduced time to recovery when in Retigabine means that they likely have an active binding site. We can hypothesize that they have channels in *C.* elegans homologous to the ones it binds to in rats and mice.

FDA approved AEDs were chosen based on the wide variety of channels and enzymes they targeted, including some polypharmacology drugs like Levetiracetam and some commonly used on their own like phenobarbital (Sills, 2011). The specific drugs chosen to test were Levetiracetam, Lacosamide, Phenobarbital and Phenytoin, the last

two being from the early 1900s (Baumann and Ryan, 1999). All of these drugs have been shown to reduce seizure duration in mammalian systems which led to them being approved by the FDA as AEDs.

Levetiracetam targets synaptic vesicle protein 2A and is a possible target for HVA Ca^{2+} channels and $GABA_A$ receptors (Sills, 2011). Lacosamide targets voltage gated Na^{2+} channels and possibly targets carbonic anhydrase (Sills, 2011). Phenobarbital targets $GABA_A$ receptors and possibly targets glutamate receptors and HVA Ca^{2+} channels (Sills, 2011). Phenytoin targets voltage gated Na^{2+} channels (Sills, 2011).

There is no perfect model system for any research, seizures and epilepsy included. It is possible that a target could be found using *C. elegans* that may not be associated with seizures in humans yet.

In this thesis, I show that *C. elegans* can potentially be used for a novel seizure model and are responsive to several AEDs that are currently in use. This method also could be used for a high throughput screen to test for novel AEDs.

Materials and Methods

C. elegans Maintenance

To complete this research, I worked under Monica Risley in the Dawson-Scully Lab at Florida Atlantic University (FAU) for project development and guidance as well as with Justin Minnerly in the Jia lab of FAU for *C. elegans* care and maintenance information. Each step requires a bottom-lit microscope to be able to see the microscopic worms clearly**.** I maintained the *C. elegans* in accordance with (Hope). When not in use, the worms were kept in a 20° incubator. I kept them on pre-made NGM (nematode growth media) agar plates seeded with E. coli strain OP50, and transferred either select

few *C. elegans* to begin another plate or transferred them by cutting out a cube of an old agar gel filled with the worms and turning that cube upside down on the new agar plate (Hope, 1999). The worms were deemed ready when they were in the L4 stage.

C. elegans Preparation

Once worms were in the L4 stage, they were picked up using a thin platinum wire melted into a glass pipette and placed onto a separate agar plate on their own with bacteria. The platinum wire was flamed before touching the original plate again. Once this plate was adequately full, they were left in the incubator for 24 hours (Hope, 1999).

After 24 hours, I took the worms out of the incubator to prepare the assay for inducing electroconvulsive seizures. To achieve this end, thin plastic tubing was cut into 9mm long pieces and with the use of a pipette, were filled with M9 saline solution or a pre-made mixture of the M9 saline and a drug to supply conductance. Once the tube was ready, five to ten *C. elegans* were added to the tube using the same platinum wire method as before, flaming the wire in between each touch back to the original plate (Hope, 1999). The worms were allowed to incubate in the solution in the tube for 30 minutes for each trial before they were ready for testing.

C. elegans Antiepileptic Drug Testing

After incubation, two single gage copper wires corresponding to positive and negative stimuli attached to a square-pulse generating stimulator were fit into the ends of the plastic tube snugly, as shown in Figure 2.

With the assay set up, a shock was delivered at a frequency of 200 hertz, duration of 3 seconds, amplitude of 47 volts and with no delay. During this shock, air bubbles often formed at one end of the tube. This process was recorded to DVDs from a camera attached to the microscope.

Data Acquisition and Analysis

The time to recovery was later calculated from the recorded DVDs to include from when the shock was initiated until the worm completed one normal body wave at any speed. Seizure was determined by a halting of normal body movement in favor of stillness or unilateral body bends as shown in Figure 3. When fitting the copper wire into the plastic tube, the copper wire occasionally inadvertently crushed some *C. elegans*, rendering them unusable for the experiment and left out of the data collection. Any worms that were obscured by air bubbles created in the tube were also left out as beginning of seizure and beginning of recovery could not be accurately determined.

Results

Statistics were found in Sigma Plot by running a Student's T-test. Figure 4 shows that there is a significant difference between *N2* in M9 and *egl-4* in M9. An n of 27 *N2* worms and 51 *egl-4* worms were used in M9. *N2* worms were shown to recover unassisted from the seizure in 64.3 seconds. *Egl-4* worms however were shown to recover in 196.3 seconds. Significance was shown between the *N2* and *egl-4* worms tested in M9 with a p of 0.001. *N2* worms were visually distinguishable from *egl-4* worms when being shocked. Both began making normal sinusoidal body movements and

both strains froze and began seizing, with the worms bending into unilateral body bends. Once they begin to recover is where they differ; *N2* worms recover rather quickly and once they recover, they immediately regain the movement they had before the shock was delivered. *Egl-4* worms begin to regain locomotion very slowly. Their body movements begin the original sinusoidal movements very slowly before eventually regaining full speed. The times were taken from when the *egl-4* worms began the slow normal movements.

Figure 5 depicts the *N2* and *egl-4* tested in M9 as well as M9 and four different drugs. Significance has also been shown between *egl-4* in M9 and Levetiracetam and *egl-4* in M9 as well as *egl-4* in M9 and Lacosamide and *egl-4* in M9. An n of 40 *N2* worms and 17 *egl-4* worms were used in M9 and Levetiracetam. *N2* worms were shown to recover unassisted from the seizure in 73.1 seconds. *Egl-4* worms were shown to recover in 103.2 seconds. Significance was shown between the *egl-4* in M9 and *egl-4* in M9 and Levetiracetam with a p of 0.05. An n of 19 *N2* worms and 27 *egl-4* worms were used in Lacosamide. *N2* worms were shown to recover unassisted from the seizure in 69.1 seconds. *Egl-4* worms were shown to recover in 92.9 seconds. Significance was shown between the *egl-4* in M9 and *egl-4* in M9 and Lacosamide with a p of 0.004.

Significance was not found with Phenytoin and Phenobarbital either with *N2* or *egl-4*. An n of 22 *N2* worms and 5 *egl-4* worms were used in Phenobarbital. *N2* worms were shown to recover unassisted from the seizure in 61.1 seconds. *Egl-4* worms were shown to recover in 106.5 seconds. No significance was shown between the *egl-4* in M9 and *egl-4* in M9 and Phenobarbital as there was a p of 0.989. An n of 38 *N2* worms and 10 *egl-4* worms were used in Phenytoin. *N2* worms were shown to recover unassisted

from the seizure in 72.8 seconds. *Egl-4* worms were shown to recover in 150.8 seconds. No significance was shown between the *egl-4* in M9 and *egl-4* in M9 and Phenytoin as there was a p of 0.098.

Discussion and Conclusion

From these results, I believe my hypothesis is supported and that *C. elegans* have the potential to play an important part in the development of novel AEDs. Significance was shown with two of the four drug trials completed and a higher n could supply further information on these results.

For the testing of *N2* and *egl-4* in M9, significance was determined. *N2* is the wild type and *egl-4* contains less PKG than *N2*. As hypothesized, due to this difference in PKG, the *egl-4* mutants are more susceptible to seizures. This shows promise for the *egl-4* worms to be used as a chronic seizure model. This successful test allowed us to continue on to try previously FDA approved AEDs to see if they help significantly reduce time to recovery.

Egl-4 in M9 and Levetiracetam was shown to have significantly decreased recovery time in comparison to the *egl-4* in M9 control. *Egl-4* in M9 and Lacosamide was also determined to significantly decrease the recovery time against the *egl-4* in M9 control. Lacosamide targets voltage gated Na^{2+} channels and possibly targets carbonic anhydrase. There are no Na^{2+} channels in *C. elegans*, so it if this is the targeted effect, there is most likely a side mechanism occurring to show an effect.

Phenytoin and Phenobarbital were not shown to significantly decrease recovery time. The n for these two drugs with *egl-4* in M9 and drug solution was on the low side and a higher n could reduce the error bars and give a clearer picture of what is happening.

For any these drugs, it is also possible that the duration of exposure to the *C. elegans* was not enough at only 30 minutes to cause a significant effect. A duration curve could be determined with each drug to determine the most useful incubation time. It is possible that a longer incubation time can show an increased significance in Lacosamide and Levetiracetam.

In this thesis, I have shown the potential of *C. elegans* as an electroconvulsive model system. Future directions on this project would be to create a high throughput assay to be able to test more AEDs at one time and to create tracking software for quick and accurate analysis of the time to recovery.

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Figures

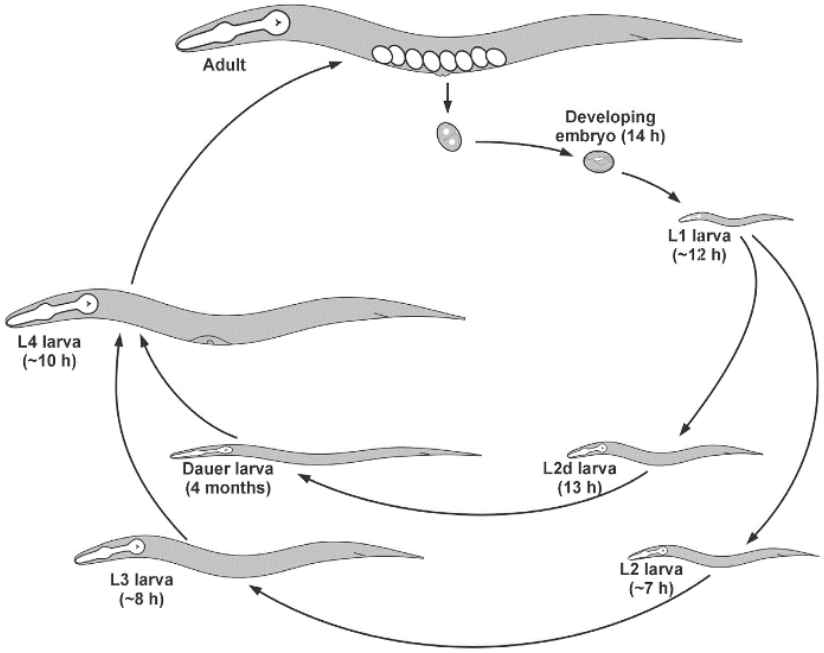

Figure 1. The life cycle of Caenorhabditis elegans (Strange, 2003).

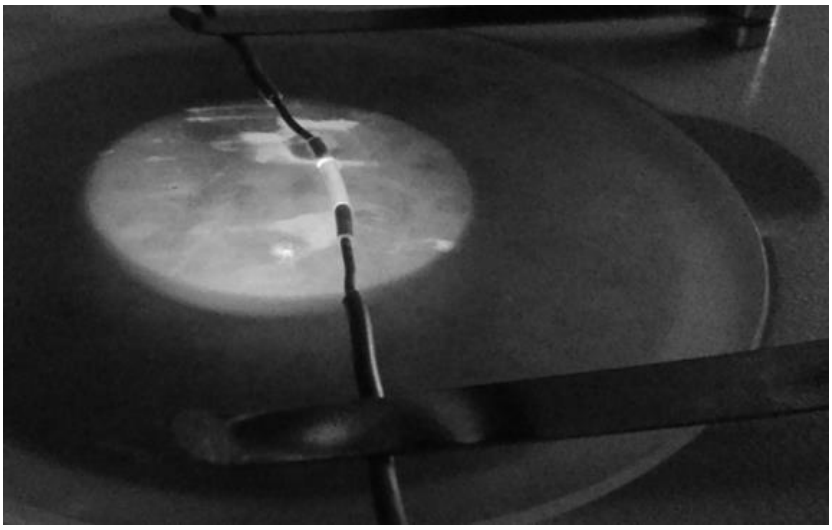

Figure 2. The electroconvulsive assay including the 9mm plastic tubing filled with M9, *C. elegans* and plugged with copper wires attached to an electric stimulator.

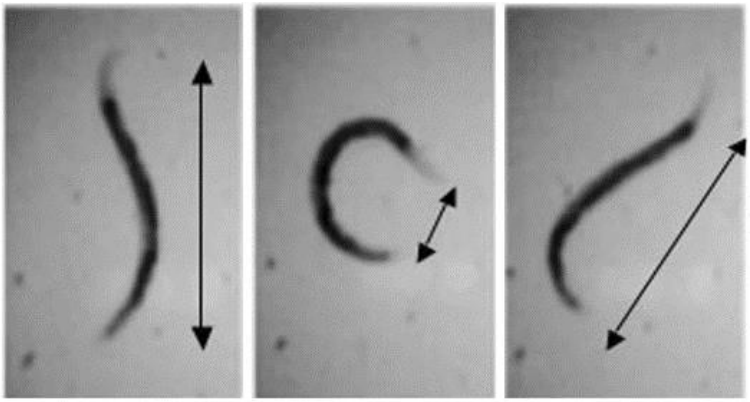

Figure 3. *C. elegans* completing a unilateral body bend as often depicted during electroconvulsive seizure (Hart, 2016).

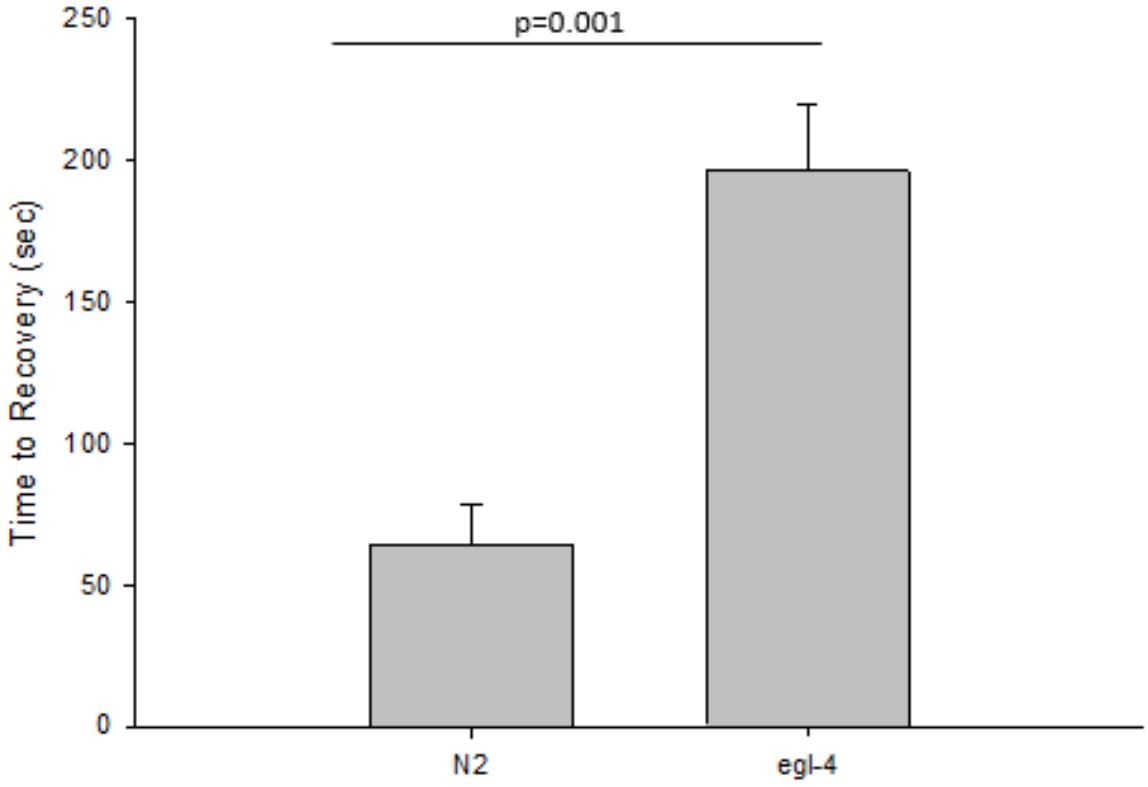

Figure 4. *C. elegans* genetic strain variation comparison.

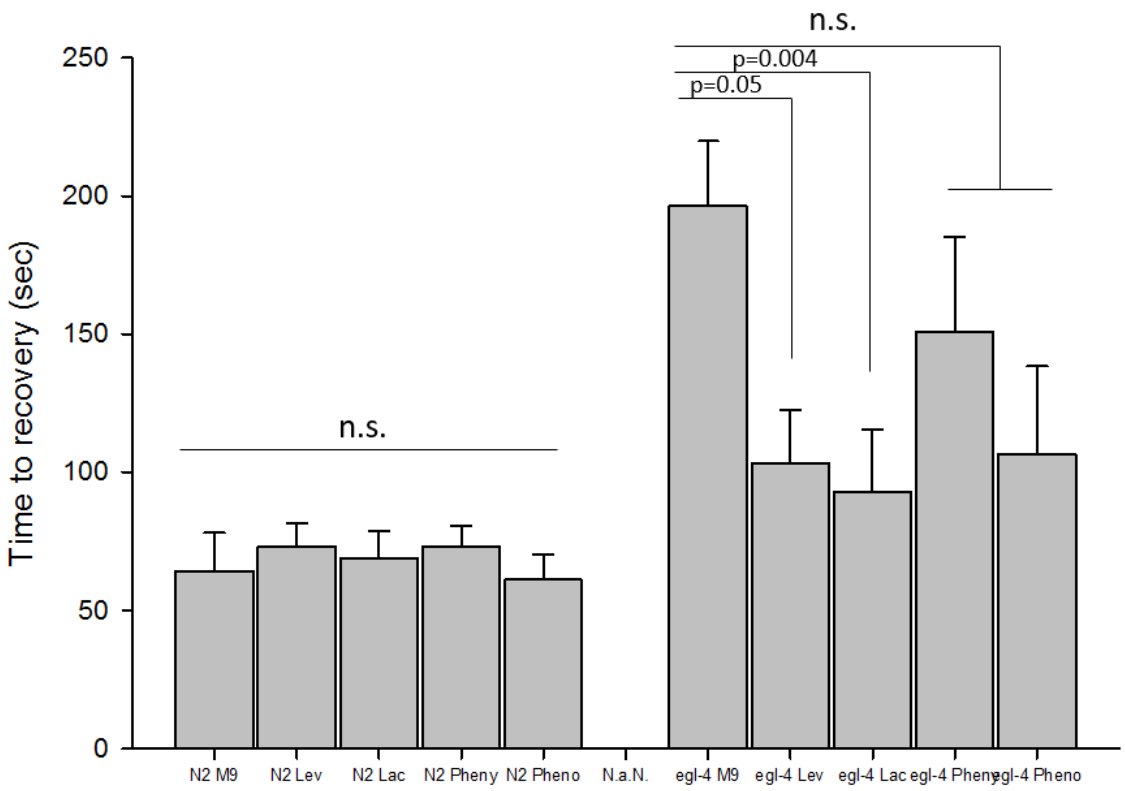

Figure 5. *C. elegans* pharmacology