The Effects of Stress Stimuli on Pmec-4 *Caenorhabditis elegans* Mechanosensory Neurons

A Major Qualifying Project Report

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This report represents the work of one or more WPI undergraduate students submitted to the faculty as evidence of completion of a degree requirement. WPI routinely publishes these reports on the web without editorial or peer review.

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1. Abstract:

Various natural mechanosensory stress factors have been studied as factor in the pathogenesis of different neurodegenerative diseases. Different mechanosensory stimulus leads to the alteration of protein signaling pathways that result in the degradation of neurons. The effects of stress on the bodies and neurons of worms are very similar to the same physical effects seen on human cells. This study conducts two separate experiments to observe how neurons can react to stress at different stages of life using a transgenic strain of *Caenorhabditis elegans* with green fluorescent protein tagged to the MEC-4 protein associated with soft touch stimulation. The first experiment looks at overall activity of the soft touch mechanosensory neurons following stress. The second experiment looks at trend in morphological abnormalities following the same stress stimuli. We saw that adult worms have more resilience against osmotic stress and recover function after a time lapse of stress stimulus. Younger stage worms show the opposite effect when flooded with excess acetylcholine. There were distinct changes in morphology of both the somatic body and axon of the select soft touch neurons (ALML) that could be characterized into five categories: pilling, somatic deformation, gastrointestinal bleeding, axonal straightening, and axonal waviness. These changes indicated intracellular damage result in swelling of the cell and alterations in membrane tension from mechanical stress.

2. Introduction:

There are many neurodegenerative diseases that can be linked to the natural aging of neurons. Neurodegeneration refers to the process where neurons progressively atrophy and lose function. There are many causes to neurodegeneration, but scientists speculate that it is induced by stress stimuli leading to aggregation of proteins into plaques that turn into neurofibrillary tangles (NFTs) which disrupt signaling function (Glenner and Wong, 1984). *Caenorhabditis elegans* have been used in a number of studies as a way to study the pathophysiology of various neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS). Strains have been developed to express targeted proteins associated with these disease pathways (Link et al, 2006).

Scientists have found evidence that the disruption of G-protein coupled receptor (GPCR) pathways is indicated in multiple neurodegenerative diseases. The GPCR, DEG/ENaC (degenerin/epithelial Na^+ channel) are indicated in the transduction of mechanosensory signals (Corey, 1997: Goodman, 2004). The MEC-4 channel is thought to play a role in the conduction of soft touch stimulus. Alterations in these pathways can occur from induced mechanosensory stress factors. As degeneration occurs, the overall activity of the neurons is depleted. Under microscopic conditions it can be observed that alterations from the cell's normal morphology can be a hallmark for cellular dysfunction seen the atrophy stages neurodegenerative conditions (Yagishita, 1978).

3. Background Information:

In aging studies *C. elegans* are an ideal model organism for human diseases involving degeneration. Worms evolve and grow at a rate that can be compared to the human life cycle (figure 1). *C. elegans* are a microscopic organism in the Nematoda phylum. They are used a model organism for many studies due to their similarity to the human body. C. elegans contain a nervous system much like the human body, with a nerve ring acting similar to a brain. The structure of their outer cuticle mimics the consistency and biochemical makeup of human skin. Due to their short life expectancy, fecundity, and low cost, they are an ideal model for many diseases that can be found similarly in humans. Although they lack many of complex body structures humans have, they have a mouth and digestive system, the ability to reproduce, a muscular system, and the ability to sense chemicals as smell, taste, and touch via heat detection. Although we originally believed C. elegans were naturally found in soil, they are more likely to be found on rotting fruit. Many transgenic lines of wild-type strains of C. elegans have been generated in order to target specific genes and their functions, by tagging different fluorescent proteins such as GFP to targeted sites that will illuminate different biochemical pathways when stimulated.

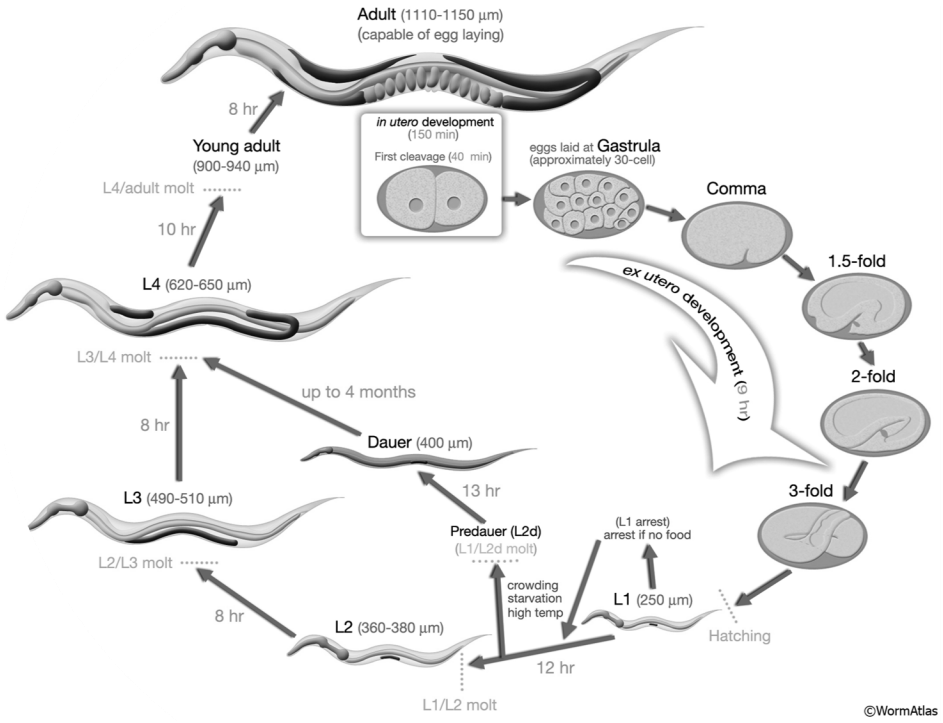


Figure 1: The life cycle of a hermaphrodite *C. elegans* worm. The worm starts as an egg where it hatches into an L1 worm. The worm maintains four larval stages until it grows reproductive organs and matures into a fertile adult. The stages of development can be thought of as reflective of the human life cycle. The egg would be considered a baby, while the larval stages are considered early development into the adolescence. The adult stages coincide with adulthood in humans (20 years old and above)

For these transgenic lines, genes that are connected to ion channels are often targeted. The zdIs5 strain (Clark et al, Driscoll et al) target the mec-4 degenerin channel in C. elegans. Degenerins are a family of proteins that are known for having single mutations that cause degeneration of connected neurons due to disruption of the cellular membrane that leads to lysis and cell death. The mec-4 channel is connected to the mechanosensory system, that is connected to the six low threshold touch stimulus neurons (AVM, PVM, ALMR, ALML, PLMR, PLML) that extend along the body of the animal. These are microtubule cells that contain processes with 15-protafilament cross linked to each other, so their distal ends are near the cell membrane (Chalfie and Thompson, 1969). Mec-4 (Mechanosensory abnormality protein 4) is a subunit of a mechanically gated amiloride channel (sensitive to sodium ions), that is responsible for transduction of touch stimuli in the animal. Mec-4 mutants are known for exhibiting neurodegenerative phenotypes. The strain used in this study, zdIs5, is generated in the Driscoll lab at Rutgers University has been produced to tag the six mechanosensory neurons with a GFP protein tagged to the MEC-4 protein known to be responsible for ligand-gated sodium channel activity involved in the detection of mechanical stimulus. MEC-4 is known to be located on the cell membrane of both the axon and neuron projection. The strain of worm was developed as a transgenic model for both ischemia and neurodegenerative disease (figure 2).

4. Materials and Methods:

4.1 Worm Strain:

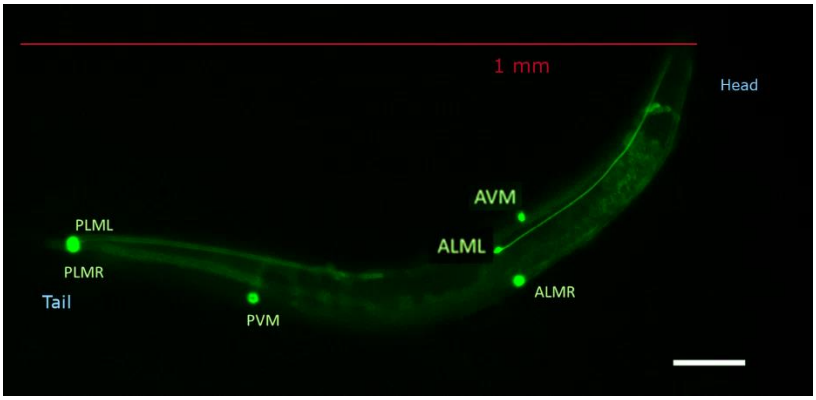


Figure 2: For this experiment, we used the C. elegans strain ZB154 zdIs5[mec-4::gfp] provided by the Driscoll Lab at Rutgers University. This strain has been produced in order to image the six mechanosensory neurons of the worm (PVM, AVM, PLMR, PLMR, ALML, ALMR). Mec-4 strains are known to show neuronal degradation over time due to their nature as degenerins.

4.2 Worm Maintenance:

Standard protocols were used to maintain the worms. Worms were stored in 20 degrees Celsius and transferred onto E. coli seeded plates to grow. After eggs hatch, we waited 44 hours for the L3 molt to occur and 65-128 hours for the worms to reach the adult stage. Worms were transferred using a worm picking tool (a glass pipet and bent wire) onto an unseeded plate. Worms were then treated with 1 µL of a paralytic agent and transferred onto a preassembled agar padded glass slide.

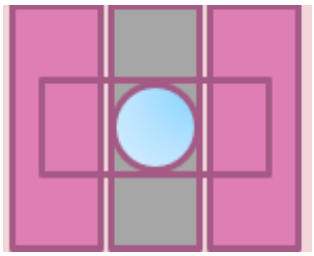


Figure 3: Agar pads were assembled using four glass slides and a drop of agar. The two outer slides were lined with tape to raise the cover slide. 1 % agar was melted on a hot plate and transferred with a pipet onto the middle slide. The drop of agar was immediately covered with a top slide and allowed to cool. Once cool, the two outer slides are removed, and the top slide is carefully slid off.

4.3 Imaging Preparation:

Once worms were transferred onto a slide (note: did not use a cover slip) and brought to be imaged on an ISS microscope. Images of worms were taken prior to stimulus. Once a control was established, .8 µL of stimulant solution was placed onto the worms. Images were taken as a time lapse post stimulus until worms recovered or showed signs of apparent animal death.

Worms were stimulated with pre-made mechanosensory stimulant solutions. A hyperosmotic stress was induced through use of a 1 mM sodium chloride salt solution. We also applied an acetylcholine stress using a 1 mM carbachol solution (figure 4).

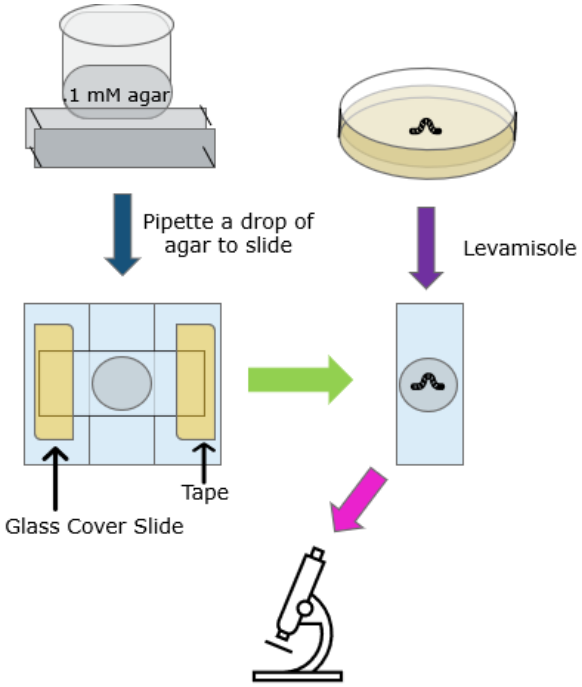


Figure 4: The second experiment required slight altercation in the initial preparation for imaging on Zeiss. The agar prepared had to be 1mM and thin enough that the light would not reflect and distort the images. The above schematic shows the protocol followed for each worm trial. The worms were picked using a micropipette and a drop of the levamisole paralytic agent. Each worm was transferred onto an agar pad slide in a damp box to prevent the worms from drying out. The worms were found using the Zeiss microscope and then a z-stack was taken as a control image. A 2ul drop of the prepared stimulus was added directly to the worm and the ALML neurons were focused on and recorded for a 10-minute time lapse. The same worm was then scanned for secondary z-stack to monitor overall changes following the stress compared to the initial scan.

4.4 Analysis:

After the images were obtained, they were analyzed frame by frame using ImageJ to assess changes in overall intensity of fluorescence. Select neurons were also measured for changes in the size of the somatic body. Most analysis for the second experiment was qualitative observation in changes of morphology before and after a stress stimulus was introduced.

5. Results:

5.1 Experiment 1:

A comparison of adult worms to adolescent in response to select mechanosensory stress stimuli.

Figure 5: Hyperosmotic stress stimulus to adult worms shows loss of function of outer neurons. Time lapse shows the six mechanosensory neurons remain active as the adult worm recovers. 3 minutes after stimulus, worm appears as before stimulus.

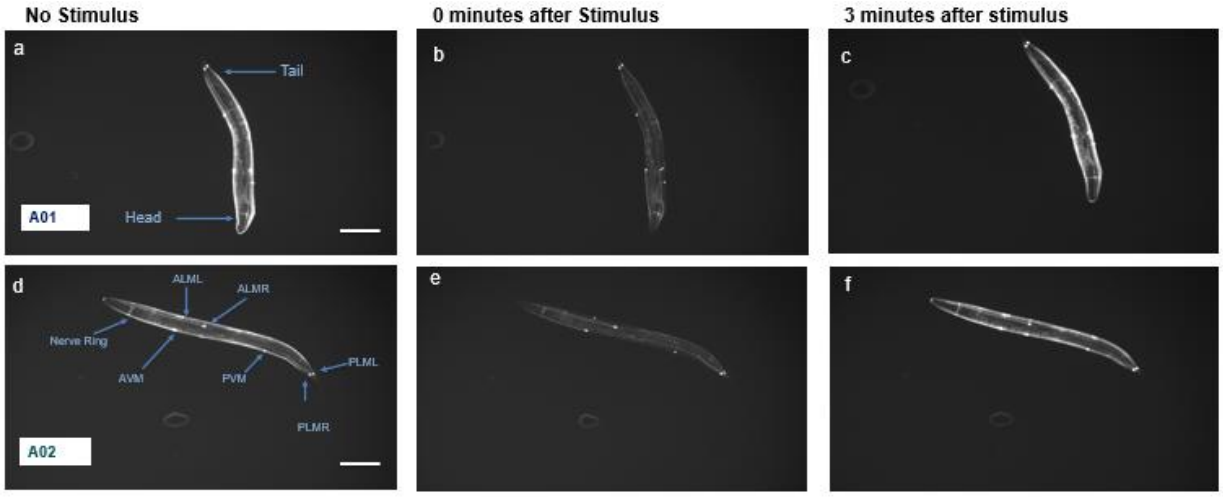


Figure 5a: Adult worm (A01) is shown to be highly fluorescent without any stimulus applied to the animal. The six mechanosensory neurons (AVM, ALML, ALMR, PVM, PLML,PLMR) are clearly being shown from the mec-4::gfp tag. Animals are showing high amounts of soft touch sensing activity even when affected by the paralytic agent. The worm initially shows straight axons and a high output of overall activity throughout the animal.

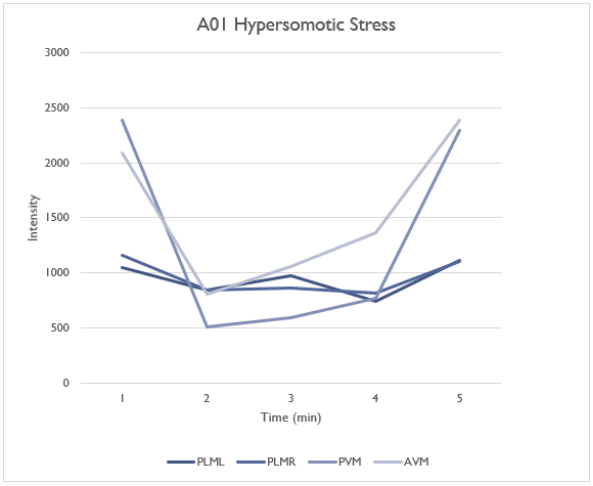
Figure 5b: A01 shows immediate loss of fluorescence following an addition of a 10 mM NaCl solution placed directly onto the worm. This indicated a decrease in activity of pmec-4. The animal s and the till exhibits activity in the six mechanosensory neurons. The PVM and AVM showed the greatest decrease in fluorescence. The PLML and PLMR lost some activity but remained the most constant for light intensity (Graph 1). Following addition of stimulus, the axons that were once straight and clear now appear to have a wavy appearance and signs of beading.

Figure 5c: After a lapse of 3 minutes following stimulus. The animal A01 exhibits signs of recovery indicated by the increase in fluorescence. The animal shows return of outer neuronal activity and increase of activity in the PVM and AVM. The animal's axons return to their straight state similarly to the original control image with no stimulus and beading and waviness appear to return to their original conformations.

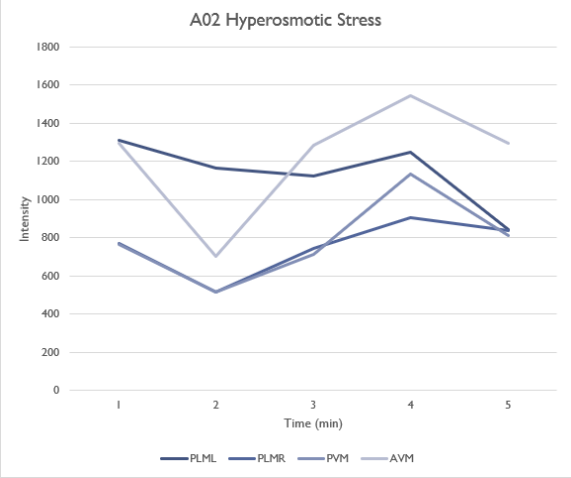
Figure 5d: Adult worm (A02) is also shown to be highly fluorescent with no added stimulus. The six mechanosensory neurons are clearly active and visible.

Figure 5e: A02 also shows immediate loss of fluorescence following an equal addition of 10 mM NaCl salt solution. Like A01, A02 shows the highest drop of intensity through the PVM and AVM, (see graph 2) while the lateral neurons remain at a fairly constant activity output. A02's axons also appear to become beaded and wavy following the loss of fluorescence.

Figure 5f: Following a lapse of 3 minutes, A02 is also shown to recover its original intensity and revert to the same light output as its control state. Axons appear to return to their original straighter state as neurons regain intensity.



Graph 1: Worm A01 with no stimulus appears to show a high amount of activity in the mechanosensory neurons, indicated by light intensity of the image captures. Following immediate hyperosmotic stimulus, the AVM and PVM show a steep decrease of light output, until recovery to original intensity is shown by the increase of intensity. The PLML and PLMR tail neurons show very little change throughout the control and application of stimulus. Like the more medial neurons, the PLML and PLMR show a decrease of activity, and then a slight increase following into the recovery period.



Graph 2: Worm A02 with no stimulus, also shows a high amount of initial light intensity (neuron activity). Following immediate stimulus of a hyperosmotic stress, the light intensity of all mechanosensory neurons drops, but then soon recovers after a lapse of 3 minutes. A02 appears to recover to a higher intensity and then once again lose fluorescence until the intensity output is equal to its original control state.

Figure 6: Hyperosmotic stress stimulus to L3 worms. Shows loss of function of outer neurons. Six mechanosensory neurons remain lit. After time lapse, worm does not recover. Worms become shriveled and neurons dim.

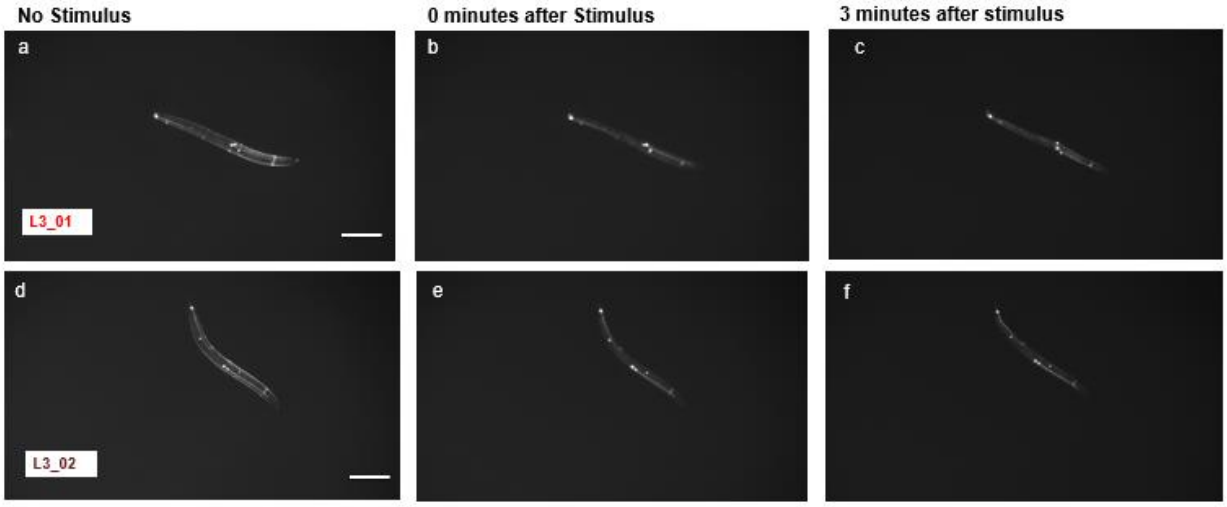


Figure 6a: Worm L3_01 is shown to also be naturally fluorescent with no additional stimulus. The six mechanosensory neurons are not shown to be quite as pronounced as in an adult. There appears to be less overall fluorescence and activity compared to the adult initially.

Figure 6b: Following immediate addition of salt solution, the L3_01 loses fluorescence in the mechanosensory neurons and outer pathways indicating loss of activity. The animal itself appears to change conformation and shrinks inward likely due to the salt solution added. The

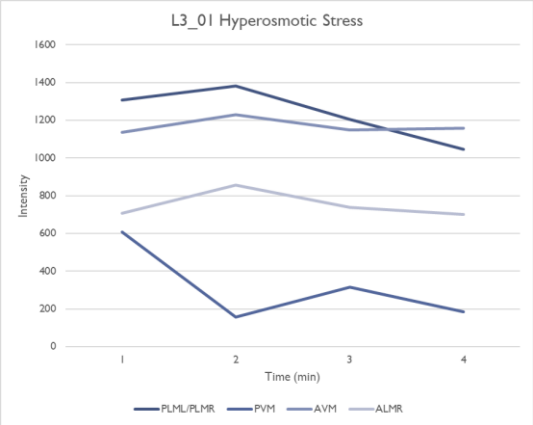
neurons appear to gain some fluorescence, but the axons become wavy and exhibit beading, indicating degeneration is occurring. All neurons but the PVM show a spike of increase of fluorescence.

Figure 6c: After a lapse of 3 minutes L3_01 continues to exhibit loss of fluorescence indicating loss of neuronal activity. The pathways that were lost are not seen to recover and the one straight axons, have now gone from beaded and wavy to a smudged like appearance. (higher magnification would allow to confirmation of whether or not the axons broke from the stress). The animal L3_01 showed no signs of recovery.

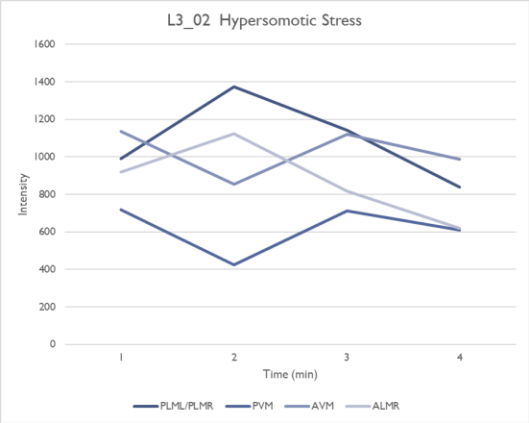
Figure 6d: Worm L3_02 is also shown to be naturally fluorescent (identical to L3_01). Like L3_01, the six mechanosensory neurons are clearly visible, but less fluorescent than the adults tested.

Figure 6e: Following immediate addition of hyperosmotic salt stimulus, the worm shows loss of fluorescence throughout the entire body. The posterior neuron (PLML and PLMR) shows a spike in fluorescence as does the AVM; however, the other neurons show immediate loss of intensity (refer to graph 4).

Figure 6f: 3 minutes after stimulus, the worm L3_02 does not appear to show any signs of recovery. All of the neurons continue to drop in fluorescence and the animal continues to shrink into itself. The beaded and wavy axons once again appeared to have fanned out into a smudge-like appearance.



Graph 3: Images were captured of worm L3_01 and analyzed to show to difference in light intensity of fluorescent output of the tagged mechanosensory neurons. L3_01 shows that initially with no stimulus, the PLML, PLMR, and AVM show the highest intensity of fluorescence. The PLML, PLMR, AVM and ALMR all show a brief increase of intensity following immediate hyperosmotic salt stimulus and show the same trend of decreasing activity. However, the PVM showed a drastic decrease in output immediately after the solution was applied. At about 3 minutes, it exhibits a slight gain of intensity, but immediately following with a loss of intensity similar to the other neurons.



Graph 4: L3_02 shows varying trends with addition of hyperosmotic stimulus. Initially, the AVM had the highest amount of fluorescence, but following the stimulus, shows a drastic drop in fluorescence, similar to the activity of the PVM. However, the ALML, PLML and PLMR both showed an increase following the stimulus, followed by a steep decline in activity. By the 3-minute marker, all neurons were losing fluorescence, and therefore showing no signs of recovery to their control state's intensity of fluorescence.

Figure 7: Carbachol stimulus to adult worms shows loss out outer mechanosensory activity. PLMR and PLML remain with highest activity.

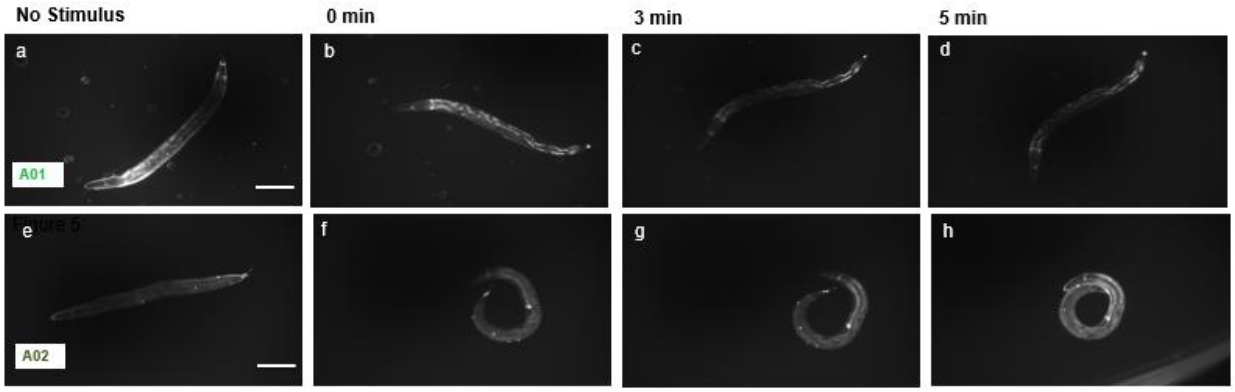


Figure 7a: Adult worm A01 shows natural fluorescence with no initial stimulus. The six mechanosensory neurons appear to not be as pronounced in late-stage adults. Worm A01 already exhibits signs of deterioration prior to addition of stimulus.

Figure 7b: Following immediate addition of carbachol to worm A01, showed a slight decrease in fluorescence in all of the neurons. Axons immediately begin to exhibit beading and waviness as the neurons began to dim.

Figure 7c: After about 3 minutes, both the anterior neurons AVM and ALML lost fluorescence to the point of almost entirely losing visual. However, the posterior neurons PLML, PLMR and PVM showed an initial increase in activity. The tail neurons PLML and PLMR showed a

constant rise in activity, where the PVM dropped very little amount activity at the three-minute marker (refer to graph 5).

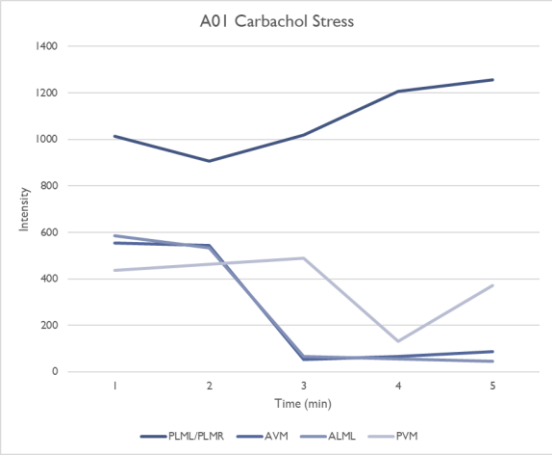
Figure 7d: Worm A01 shows no signs of recovery following the addition of carbachol. Only the PLML and PLMR are showing any increase of fluorescence and are the only neurons still clearly visible in the images captured compared to the control state of the worm with no stimulus. The worm shows visual signs of degeneration with an increase of beading and smudging of the axons.

Figure 7e: Worm A02, which is also a later stage adult shows an initial fluorescence, similar to A01, but less than the early-stage adults (Figure 2). The six mechanosensory neurons are clearly visible, indicating the animal to be at an earlier stage than the A01 control for carbachol.

Figure 7f: Following an addition of stimulus, the worm A02 shows an immediate loss of fluorescence to the animal. The outer pathways that were once expressed are no longer visible. There is immediate visual waviness of the axons throughout the worm and there was an immediate dimming of all of the mechanosensory neurons.

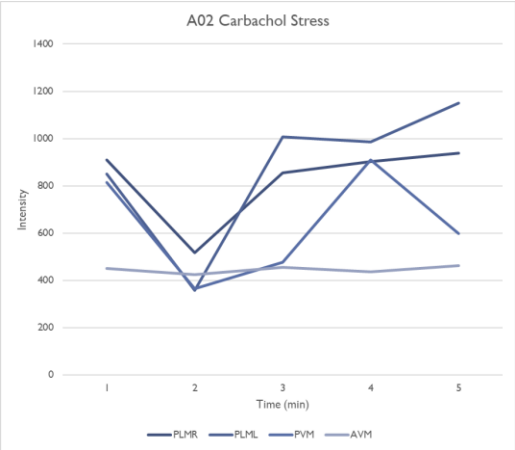
Figure 7g: A02 after about 3 minutes following carbachol stimulation shows an increase to the fluorescence of the neurons. The PLML, PLM and PVM show a spike in activity. The AVM showed little to no change in fluorescence following the addition of carbachol (refer to graph 6).

Figure 7h: Worm A02 shows slight signs of recovery of the lost pathways following stimulus. There is a spike of hyperactivity that surpasses the control state. (note: the worm curled into itself, and therefore induced its own touch stimulus. This drastically alters the results as a higher concentration of stimulus was applied to the worm. Further testing, with prevention of the change in position of the worm will be required for more accurate results).



Graph 5: Images captured of A01 for carbachol stimulation showed an immediate decrease in intensity of the neurons, PLML and PLMR following the addition of the stimulus. After a lapse of 3 minutes, the tail neurons increase their intensity of fluorescence to surpassing the amount of activity at the control state. The AVM, PVM and ALML all show a brief increase of fluorescence following the stimulus, but then have a drastic decrease in activity at about 3 minutes post stimulus. From 3-5 minutes there is a slight increase of intensity of the neurons, but

the neurons do not reach the same intensity as their initial activity output before stress was applied.



Graph 6: Worm A02, shows similar trends to the carbachol stimulated A01 post stimulus. The initial fluorescent intensity of the neurons before stimulus is very similar. However, after the addition of stimulus, all captured neurons showed a decrease of activity. Each showed a spike for fluorescence at the 3-minute marker and continued to increase intensity into the 5-minute time lapse. This drastic increase could be due to the worm curling into itself and inducing more touch stimulus than what was applied to A01.

Figure 8: Carbachol stimulus to L3 worms shows loss of fluorescence immediately after stress applied. After a time-lapse, worms begin to recover and exhibit hyperactivity of neurons until return to control state.

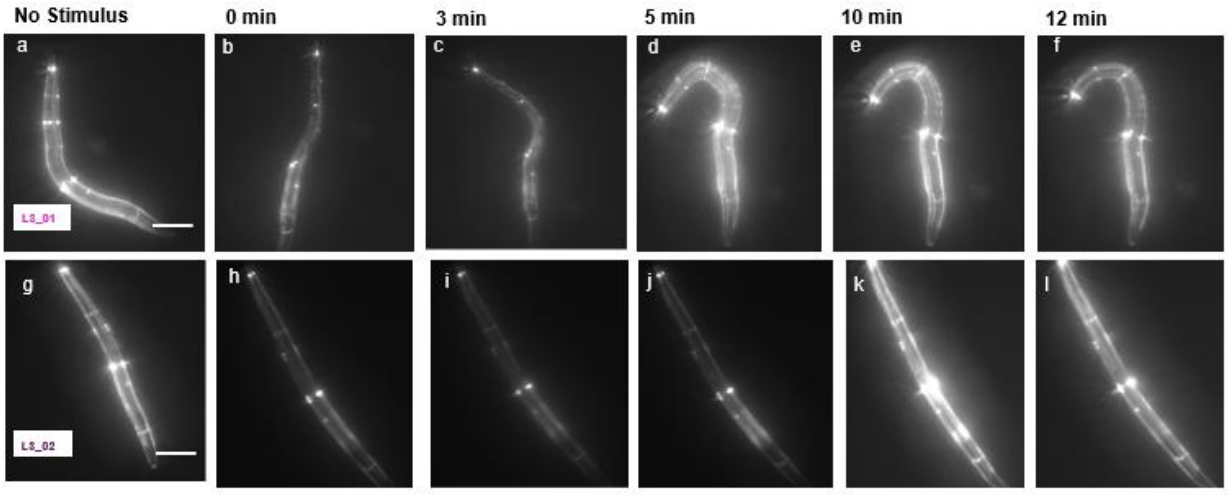


Figure 8a: Worm L3_01 shows a high amount of natural fluorescence prior to any addition of stimulus, the six mechanosensory neurons are clearly visible as are the pathways that are connected to them. The axons appear straight prior to stimulus.

Figure 8b: Following immediate addition of carbachol, the worm L3_01 shows an immediate loss of fluorescence and dimming of the neurons. There is also a visual loss of the outer connected mechanosensory pathways. The axons immediately show signs of beading and waviness, indicating degeneration.

Figure 8c: at about 3 minutes post-stimulus the neurons continue to appear to lose activity and dim. The worm L3_01 still shows no signs of recovery at this point. The axons continue to exhibit physical signs of degeneration indicated by the wavy and smudge-like appearance.

Figure 8d: At about 5 minutes post stimulus L3_01 shows an immediate spike of hyperactivity shown by increase of fluorescence of the neurons. Activity is restored to the outer pathways and the axons seem to return to their initial straight undamaged state.

Figure 8e: Following the hyperactivity and return of fluorescence, at 10 minutes the overall fluorescence of the animal begins to decrease. The axons continue to straighten to their original conformation and the neurons appear to reduce their overall activity.

Figure 8f: At about 12 minutes following the initial carbachol treatment, L3_01 appears to revert to its control state and exhibits the same fluorescent output as it did prior to the stress that was induced. The axons return to their original state and the worm shows full signs of recovery.

Figure 8g: Worm L3_02 is very similar to the other control (L3_01), showing an initial high amount of fluorescence, and a clear depiction of the six mechanosensory neurons and its connected pathways.

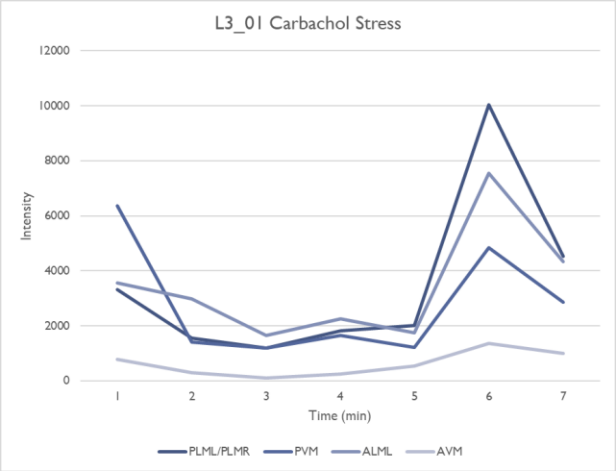
Figure 8h: After an addition of carbachol stress, worm L3_02 shows an immediate, clear loss of fluorescence throughout the animal. The once lit mechanosensory pathways on the outer worm appear to lose all activity and all of the neurons begin to dim.

Figure 8i: Following 3 minutes after the carbachol stimulation, the worm continues to drop in amount of fluorescence. The axons appear to become wavier and more beaded and only the outer neurons are appearing to show activity.

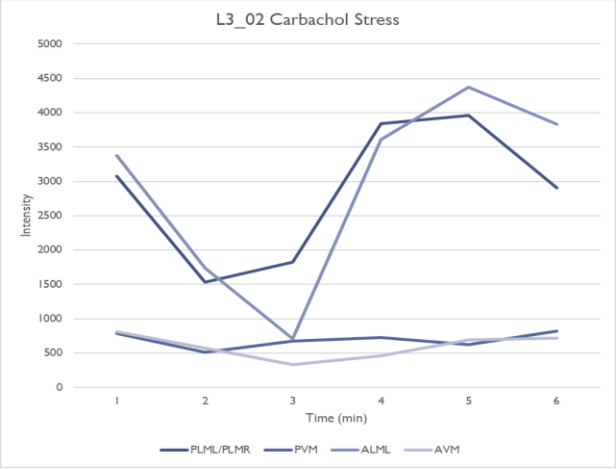
Figure 8j: After 5 minutes, L3_02 shows little change from the 3-minute image captured. There is a small increase of activity from the neurons, but the axons still appear wavy with little to no signs of recovery.

Figure 8k: At about 6-10 minutes post stimulus, L3_02 also shows signs of recovery indicated by a spike in overall fluorescence and hyperactivity of the neurons and their pathways. The axons begin to return to their original conformations.

Figure 8l: At 12 minutes after the initial carbachol stimulus, L3_02 appears to have returned to a state similar to the animal prior to the stress that was applied. The neurons have dimmed to their original intensity, indicating a return to original stress-free activity output.



Graph 5: Images of L3_01 for carbachol stimulation show an initial high output of intensity of fluorescence prior to the addition of stimulus. The PLML, PLMR, ALML and AVM all show an immediate decrease of intensity after the initial applied stress. The neurons remain at a consistent activity following the stimulus from 2 minutes to 4 minutes after. At about 5 minutes, the neurons have spike of intensity, indicating hyperactivity and a recovery from stress. At about 10 minutes, the neurons return to a lower activity output, similar to how they behaved prior to the carbachol treatment. The AVM showed the same trends as the other captured neurons but exhibited less overall activity.



Graph 6: Images of L3_02 for carbachol stimulation showed a high amount of initial fluorescence in all neurons but the AVM and PVM (interior neurons). The interior neurons showed very slight differences in activity. The lateral neurons ALML, PLML, and PLMR showed a high amount of initial fluorescence that showed a drastic decrease post stimulus. Like the L3_01 carbachol worm, L3_02 showed a spike of intensity indicating hyperactivity of the interior neurons at about 5 minutes post-stimulus. The neurons then showed a decrease of intensity until they appeared to have the same output as they did post stimulus.

5.2 Experiment 1 Summary of Results

Mechanosensory Stress Induces Axonal Degeneration and Recovery:

From the imaging data, adult worms showed loss of function in mechanosensory channel following addition of hyperosmotic stress, while touch neurons remain active. The PLML and PLMR showed higher activity during lapse of stress (Figure 5). Adult worms recovered to their control state 3 minutes after initial stimulus. Neuronal activity increases and axons regenerated to original form). It was seen that L3 worms exhibit same initial activity with no signs of recovery following stimulus with salt (figure 6). Adult worms that were treated with carbachol showed axonal degradation immediately following stimulus. Axons become beaded and begin to break. After five minutes, worms are not shown to be recovering and activity remains low (Figure 7). L3 worms exhibit loss of mechanosensory activity. After about 6 minutes, worms regained activity and produced higher activity than before stimulus. At 12 minutes, L3 worms appear as before carbachol stimulus was applied and showed higher activity (fluorescence) indicating a higher recovery rate.

Mechanosensory stress stimulus of worms with 1 mM stimulus shows immediate loss of activity of the outer mechanosensory channel. The worm loses function of all neurons but the six tagged mechanosensory neurons (AVM, ALML, ALMR, PLML, PLMR, PVM). The axons go from straight to wavy and beaded in both the adult worm and the L3. The PLML and PLMR are brightest during the lapse of time post stimulus in most tested worms.

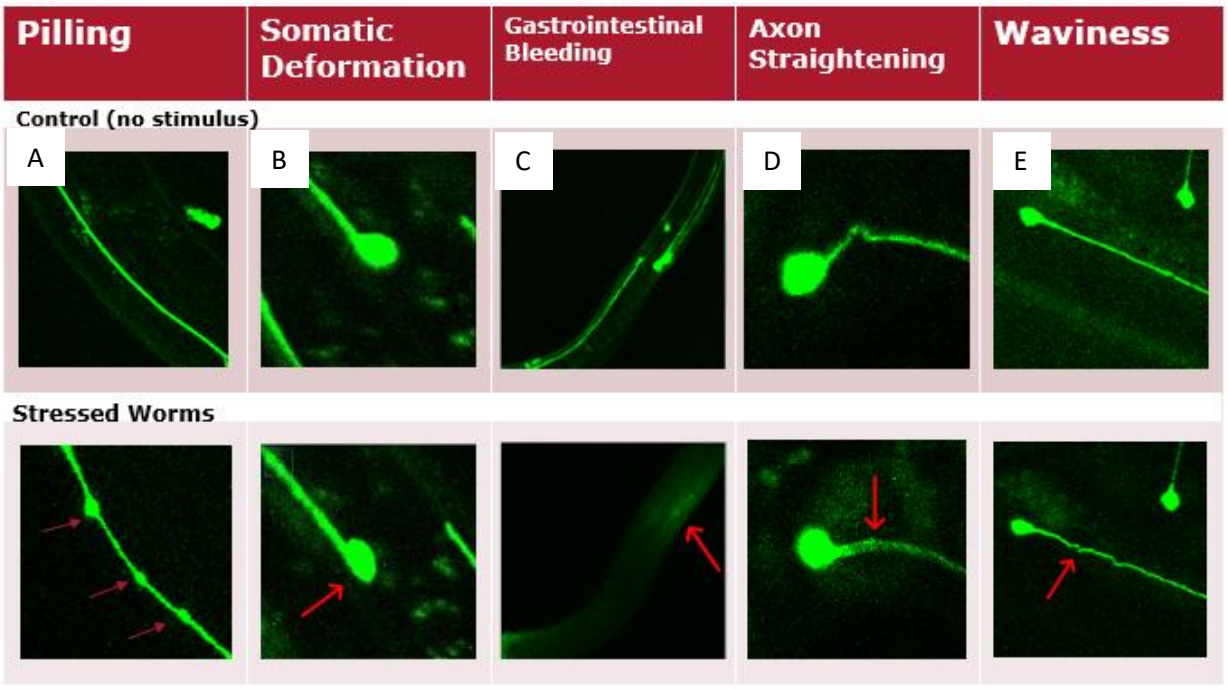
5.3 Experiment 2:

Development of a new method to characterize the age-related changes in morphology in response to stress.

Once it was determined that different life-cycle stages of the worm had varied responses to the select stress stimuli, the same strain of worm was then tested for changes in morphology that occur. This was done to determine the plasticity of the select mechanosensory neuronal cells. Various neurodegenerative diseases are caused by protein aggregation due to hyperosmotic stress. Excess number of neurotransmitters such as acetylcholine can increase cellular toxicity. We wanted to determine at what stage the worm has the highest recovery to these natural stress factors. Looking at the data, we used time-lapsed individual neurons to indicate any trends in morphological abnormalities in response to stimuli.

Using the ZB154 strain, the worms were grown in the same conditions as experiment 1. In order to have cultures that grew at the same rate, eggs were picked from bleached worms and monitored to be picked at select larval stages. The worms were transferred to pre-made agar slides to be imaged with a two-photon Zeiss fluorescent microscope. A z-stack was taken of the ALML as a control image. Once the body of the worm was imaged in slices, the stimulus (2uL) was placed directly onto the worm. That same neuron was then imaged on a 10-minute time-lapse to monitor changes in the cell body and axon. Following the time-lapse, the same cell was then scanned for a second z-stack to determine any changes the cell underwent following the stress stimuli.

Figure 9: ALM neurons showed trends in morphological alterations due to stress at all life stages of the worm.



There were five distinct morphological abnormalities that were observed by comparing the z-stack images before and after stimulus. These changes indicated the overall plasticity of the neurons and gave an indication at which stage the worm is more able to respond and recover from the stress stimuli.

Pilling/Beading:

Following the addition of stress stimuli, the axon of select neurons showed a distinct accumulation of GFP that appeared to look like beads on a string (referred to as pilling). Axonal beading is a formation of a series of swellings along the axon. This is a high indication of cellular atrophy commonly seen in the neurons of patients suffering from Alzheimer's disease (Datar et al, 2019). It is believed that the main mechanism behind this change in axon morphology is due to membrane tension occurring from the degradation of the cell's microtubules from cytosol exiting. There are two current hypotheses to this phenomenon. The first is the "traffic jam" hypothesis which suggests that beading occurs from the accumulation of organelles that happens from the breakage of microtubules (Stokin et al). The second hypothesis is that beading is induced from sudden injury or changes in osmolarity in the extracellular space that result in inflammation of the cell.

Somatic Deformation:

Neurons that were stressed showed changes in the somatic body of the cell. These changes in morphology focused only on the cellular body of the neuron. These changes in the

soma could have resulted in the cell swelling, alterations in membrane tension, or changes in overall plasticity following injury from the external stress stimuli.

In the deformation of the soma, there were some cells that exhibited the formation of novel outgrowths from the cell body directly following the stress stimuli. It is known that aging neurons can sometimes produce these outgrowths that are associated with mitochondria at their growth sites (Pan et al, 2011, Tank et al ,2011). Novel outgrowths are seen as more likely to occur in neurons that have similar beading either at the dendrites or axons. Their exact mechanism in neuronal plasticity is still unknown in association to neurodegeneration.

Figure 10: Novel outgrowth in the soma of carbachol stimulated L3 worm

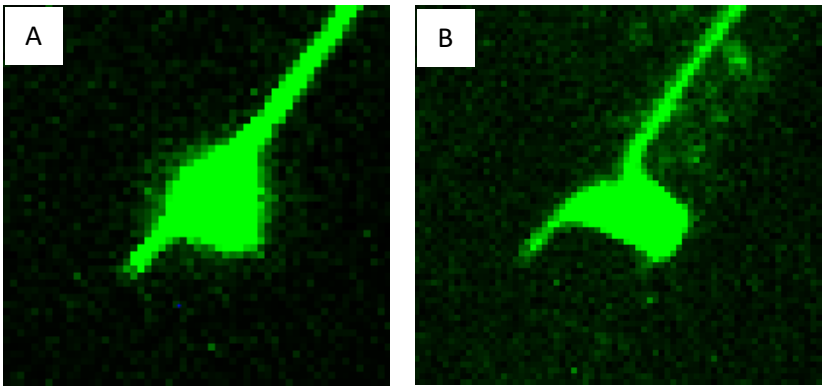


Figure 10a: Initial z-stack of the ALM neuron showed a distinct outgrowth from the somatic body of the cell looking like an extension of the axon.

Figure 10b: Following carbachol stimulus, there was a distinct change in the morphology of the cell body and a retraction of the identified outgrowth into the cell body.

Gastrointestinal Bleeding:

When the mechanosensory stimulus was added to the worm, there were often times that during the time lapse, the worm appeared to swell and 'bleed' until the neurons went from easily depictable structures to smudges across the body of the animal. We hypothesize that this drastic change in worm was due to the animal intaking the liquid stimulus into its mouth, likely leading to it entering and severely injuring the worm's gastrointestinal tract. Previous studies looking at *C. elegans* as a drug model saw this same phenomenon and measured it as an indication of shortened life span of the worm (Weeks et al, 2010).

Axon Straightening:

When the initial z-stack of the worms was taken, some showed axons that already had distinct kinks and waviness. When the stimulus was added, those axons stretched, and the kinks straightened out. This was likely due to changes in membrane tension. Axons are known to contract and straighten as a response to mechanical stress.

Axon Waviness:

A similar but opposite phenomenon occurred to the axons where following the stimulus, the axon formed kinks and ‘waves’ following the time-lapse but showed indications of recovery following the initial stress. There have been multiple studies that indicate this could be a response to injury or stress that causes mechanical retraction of the microtubules in the axon due to increased mechanical tension of the membrane (Shao et al, 2019).

Figure 11: Induced stress from excess acetylcholine showed profound changes in morphology of the ALM neuron in L4 worms.

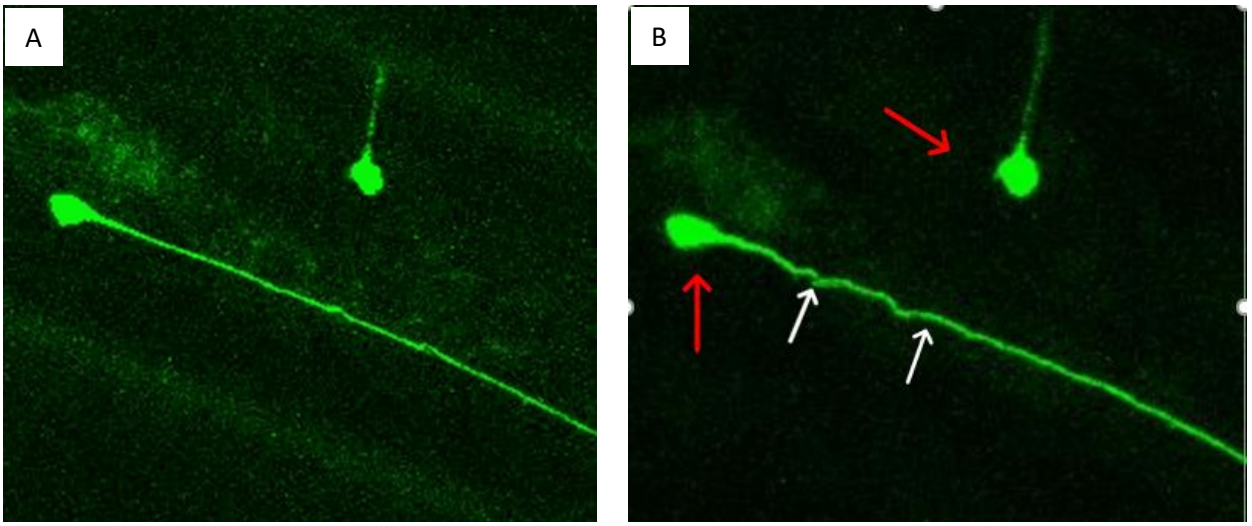
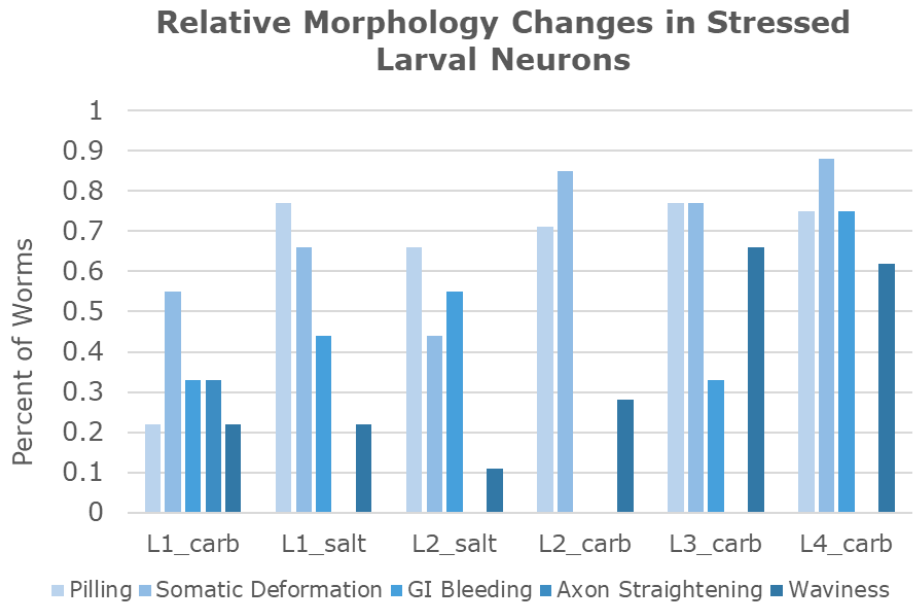


Figure 11a: This is the initial z-stack of the unstimulated worm showing the ALML and AVM. The worm appears to have a mostly straight axon and round somatic body.

Figure 11b: This is the same cell following 2ul of carbachol stimulus. The time lapse showed immediate retraction of the axon resulting in the kinks indicated by the white arrows. During the same time lapse, there was a clear change in the soma of the ALML that likely occurred due to the change in membrane tension from the mechanical stress. The AVM neuron also showed an extension of a novel outgrowth which may have accounted for the decrease in change of the soma’s morphology. After the initial kinking, a time lapse of 12 minutes showed the cell to start recovering by the re-straightening of the axon.



Graph 7: Worms were grown for select life-cycle stages and monitored for changes in morphology following induced mechanosensory stress of carbachol (ACh) and potassium salt (KCl).

The worms were grown and selected for trials at each larval stage. Starting with the youngest worms, (L1) there were distinct patterns of all five identified morphological changes (figure 9). 50% of the youngest worms treated with carbachol had somatic deformation. They also more commonly had GI bleeding and axonal straightening. L1 worms that were treated with salt had higher instances in changes in morphology. About 75% of the worms showed distinct pilling, 65% showed somatic deformation, and 45% showed GI bleeding. There were no instances of axon straightening with the L1 worms treated with salt.

The L2 worms treated with salt showed similar results. There were about 65% of worms that experienced pilling of the axon and 55% that had GI bleeding. Some trials showed somatic deformation. Like the L1 worms with salt, there were no worms that showed axonal straightening. However, the L2 worms treated with carbachol reacted differently to the stress. There were very high instances of pilling and somatic deformation. The L2 worms were unique in having no GI bleeding when treated with carbachol.

The L3 worms were only treated with carbachol and showed 75% of worms with pilling and somatic deformation. There also was 65% of worms that exhibited kinks and waviness in the axon following stimulus. The L4 worms were also only treated with carbachol and showed the highest degree of morphology changes following stress. 90% of worms experienced somatic deformation and 75% had both pilling and GI bleeding. 60% of the L4 worms showed distinct waviness, but none of the worms showed any axonal straightening.

5.4 Experiment 2 Summary of Results

L4 older adolescent worms showed a higher degree of deformation.

The younger worms that were treated with carbachol showed the highest recovery rate from the stress (specifically the L2 worms). This indicates that these younger larval worms have a greater ability to alter their neuronal plasticity in order to respond to mechanical stress. The older L4 worms showed the highest degree of deformation in both the soma and axons. The percentage of worms that showed GI bleeding indicated that they had the shortest lifespans following the stress with little ability to recover and regenerate following injury from the extracellular stimuli.

6. Discussion:

6.1 Discussion of Results

The following experiments were conducted with the goal to determine how plasticity of neurons evolves with age. Aging has been indicated as one of the highest risk factors for developing neurodegenerative diseases. There have been numerus studies exploring the causes of neurodegeneration in neurons. Our lab has previously conducted research using a PC12 rat adrenal neuronal cell line to determine which factors of stress induce changes in morphology in healthy cells (specifically axonal retraction following stimulus). With an immortal cell line however, there is no indication of how the neuron's age can alter its response to stress that naturally occurs in the body. *C. elegans* are a highly desirable model for aging studies due to how their life cycle and physiology can be comparable to that of humans. *C. elegans* show a similar decline in anatomical and functional features of their neurons as they get older including tissue integrity, motility, neuronal plasticity, and immunity (Zhang et al, 2020). These alterations in stress response are due to age-associated changes in expression of microRNAs and stress-responsive genes (Son et al, 2019). As the worm ages, it is less able to adapt to changes in its environment, including external and internal stress factors.

The ZB154 strain used in this experiment was grown on nematode growth media (NGM) and fed with *e. coli.* For the following experiments, the worms were bleached so growth of the new offspring was synched from the time the worms hatched from eggs. The worms were maintained for the duration of the experiment. The preparation of the agar varied on the microscope used for each experiment. In the second experiment 1 mM agar was the most optimal for viewing the worms at each life stage. Thicker agar was able to be used in imaging the adults in experiment 1.

There have been various imaging studies on *C. elegans* neurons. However, due to the specificity of the ZB154 strain there are few studies that use this exact strain outside of its first development as a transgenic strain in the Driscoll Lab. To our knowledge, there are also no studies that used our exact imaging techniques for monitoring morphology. Most live imaging of C. elegans has the worms either active or permanently fixed to the plate. Our method allows us to take before and after images of the same worms to show exact alterations in morphology following treatment. In order to achieve this, we used the thin agar, identified worms by putting them into distinct sections of the pad (divided the pad into two sections to have two worms per slide). Once a worm was identified, z-stack images of it were taken with Zeiss at 75 frames. In order to treat the worms, we had to directly apply to liquid stress stimulus. In earlier trials we found that when directly putting a drop of liquid onto the worm, it often would float away due to it not being entirely fixed to the agar. We were able to overcome that limitation by putting the 2ul drop on a cover slip and then spreading the drop using a cell spreader. The slide was then directly put onto the worm, ensuring full coating of the liquid (results were varied if the full worm body was not saturated). The plane of reference was then readjusted to image the selected ALML neuron for the allotted 10-minute time lapse. Following stimulus, the worm was then imaged as another z-stack to compare the stimulated worm to the initial controlled scan. The

control may not have accurately been represented as a control due to the unknown effects of levamisole. Levamisole was necessary to inhibit the worms for moving and activating their mechanosensory neurons outside of the detection of stress. In future experiments, this would need to be addressed to see if the levamisole had any effects on the initial morphology. Due to the length of time it took for each individual worm, each condition had only about 9 sufficient worms that could be analyzed with clear images.

6.2 Limitations and Future Implications

There were many limitations to this experiment. We could only speculate what was happening to the proteins within the cell and how it would swell following stress. The magnification for the microscope was not high enough to determine the intracellular processes and we relied on the observation of morphology of the entirety of the cell. Future experiments would look to observe MEC-4 protein migration and calcium signaling in response to the same stress stimuli. We would also look to have a higher number of worms imaged in each trial to validate the previous results. The following morphology chart can be used for reference in comparison of quantitative data. Conducting analysis on whether or not MEC-4 protein is aggregating in the cell will also be important to show its role in the disruption of cellular signaling. Due to time constraints, there were no results for the L3 and L4 osmotic stress stimulated worms. We require the same experiment to be ran in these conditions as well as on different stages of adult worms to show the changes in cellular function between all ages of the worms to have an accurate comparison to the human aging process.

Conclusion:

There is a definitive link to the natural aging process and the incidence of neurodegenerative diseases. As our neurons age there is a decrease in their functionality and plasticity, leading to the inability to adapt and recover from stress stimuli. We conducted two experiments looking into the *C. elegans* life cycle and how at different stages the soft touch neurons are able to react to stress stimuli. It was observed that each time the cells were stimulated, there were distinct morphological changes likely caused from alterations of membrane tension and swelling of the cell during the process of lysis in atrophy. Our data showed that the younger worms had a higher amount of adaptability with both forms of stimulus. The most profound changes in morphology were seen in the later stage L4 worms and in the stimulated adult worms. Theis could be an indication of the younger cells' ability to regenerate after injury by utilizing its natural plasticity. Most neurodegenerative diseases occur in later stages of life. The older worm neurons showed a similar increase of incidence of dysfunction at later stages of life showing that this loss of function and inability to recover following natural stress could connect to the pathophysiology of various diseases.

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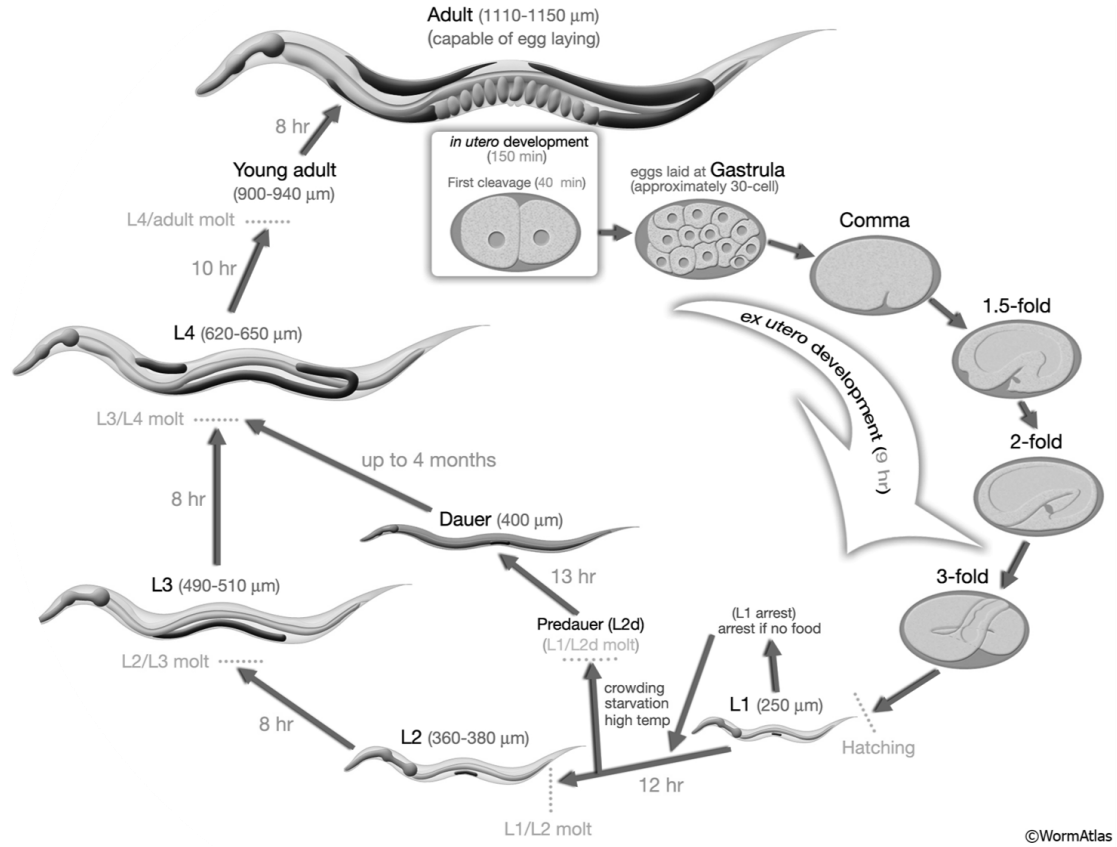
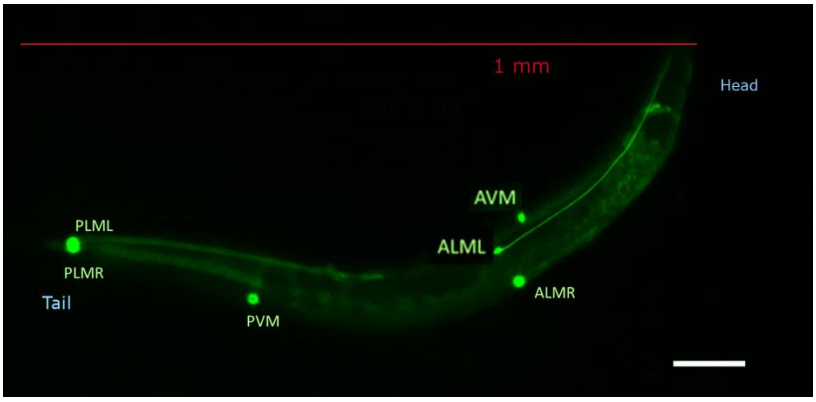
Supplementary Materials:

1. Worm Anatomy





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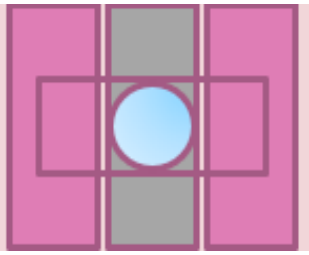

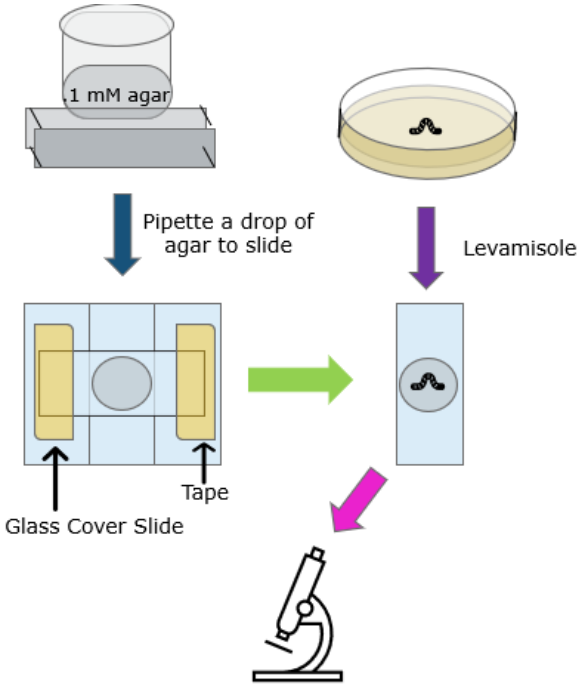

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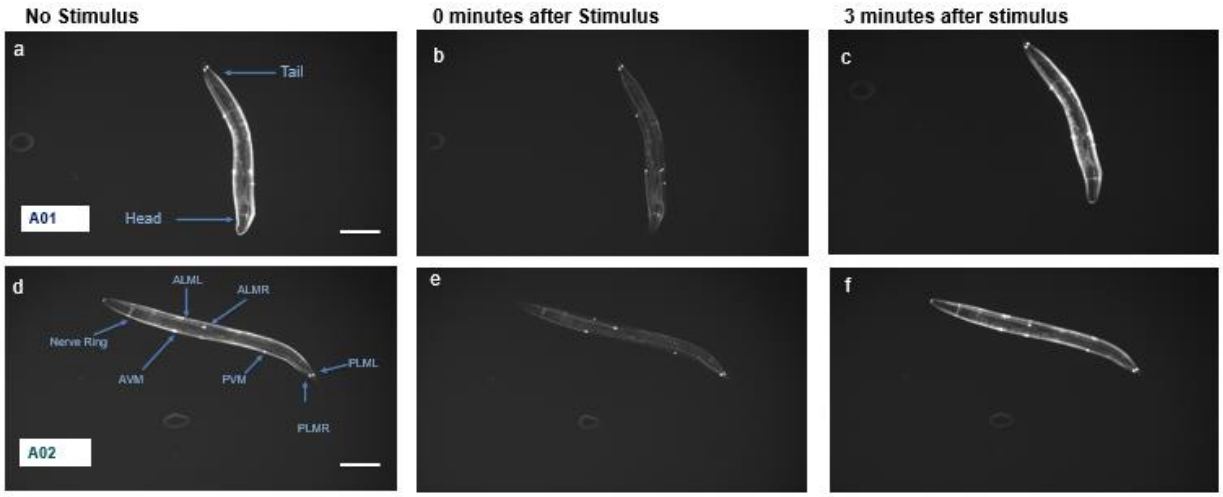

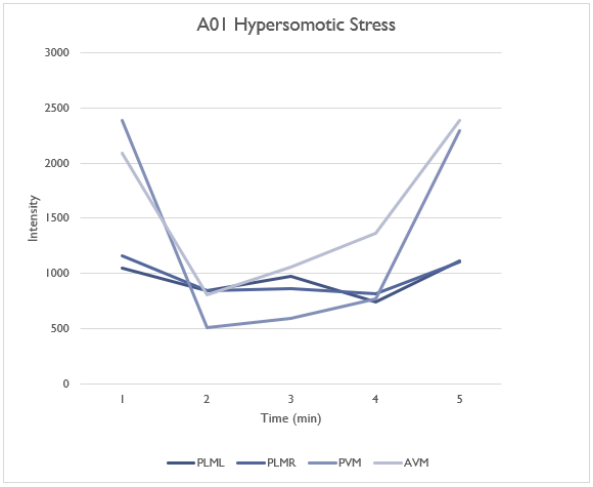

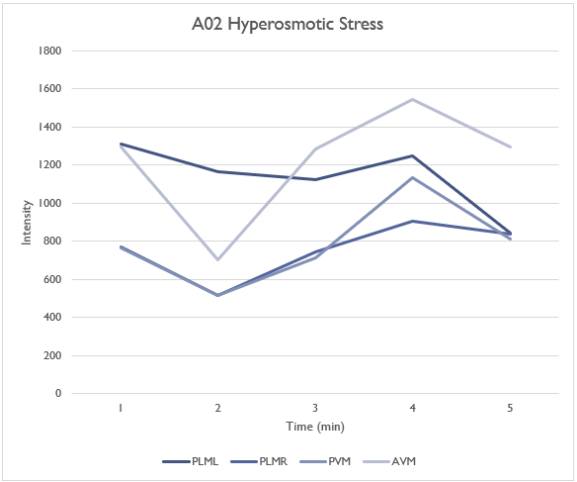

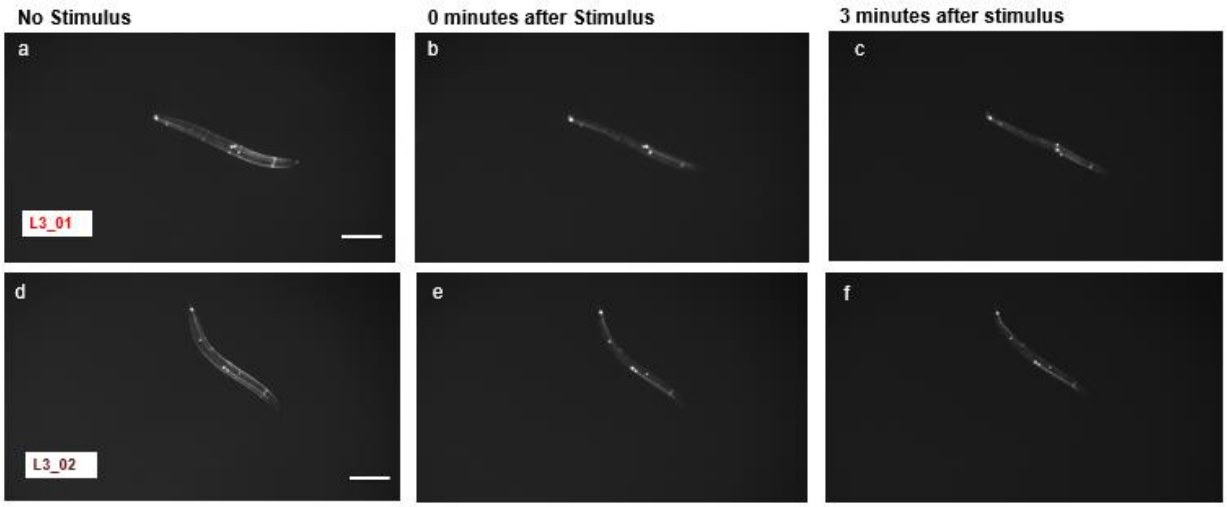
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L3_01
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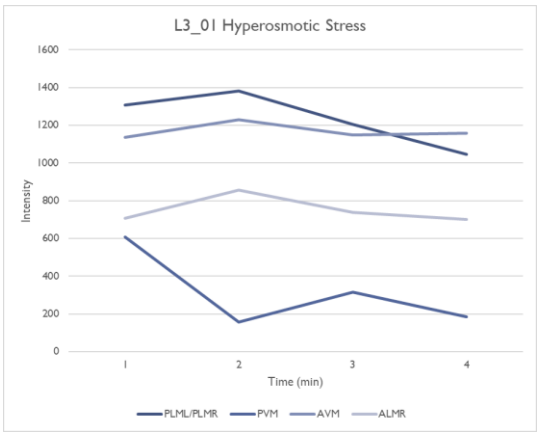
f
L3_02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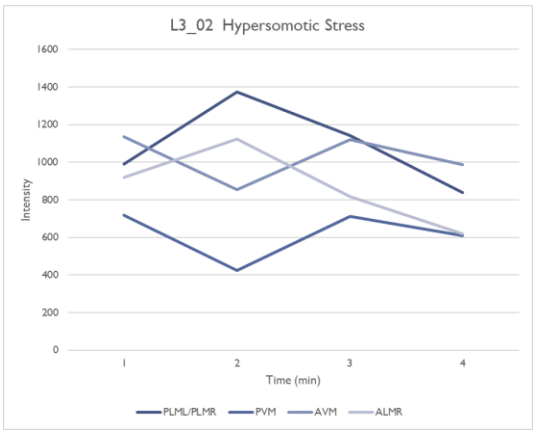
PVM
AVM
ALMR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AVM
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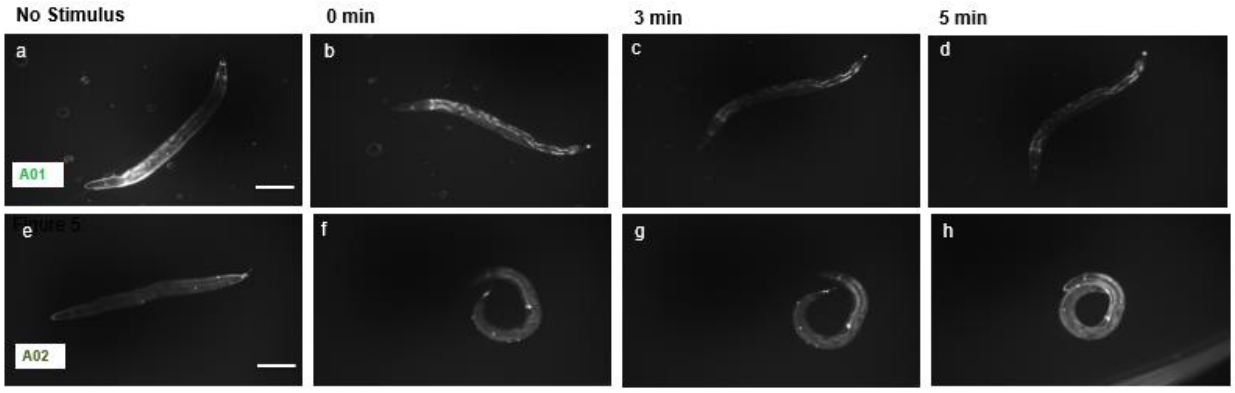
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A01
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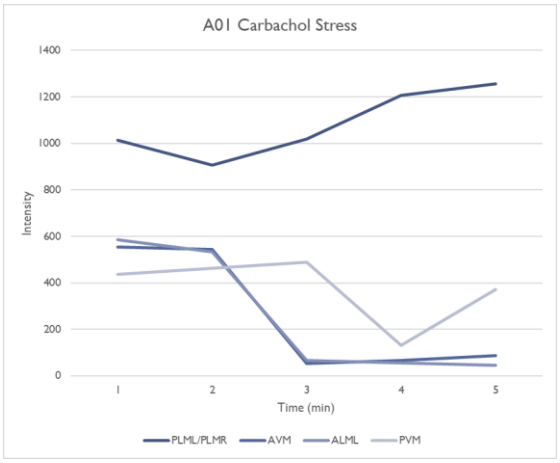
A02

Figure 5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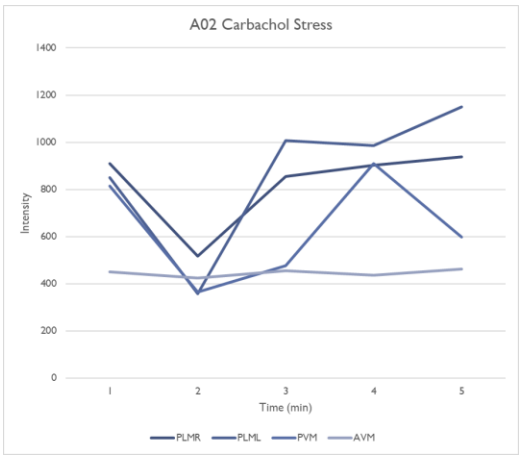
AVM
ALML
PVM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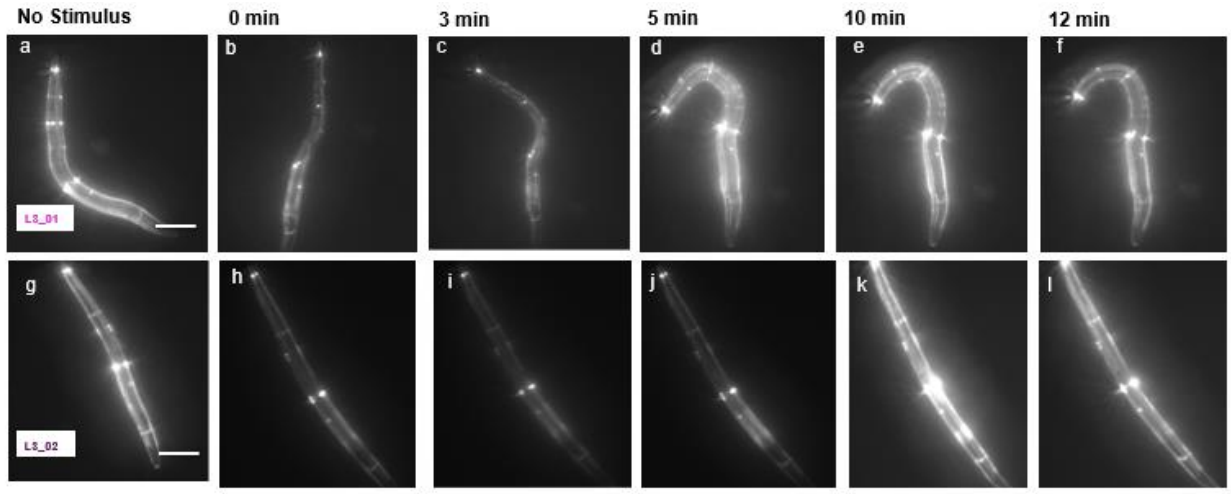
PVM
AVM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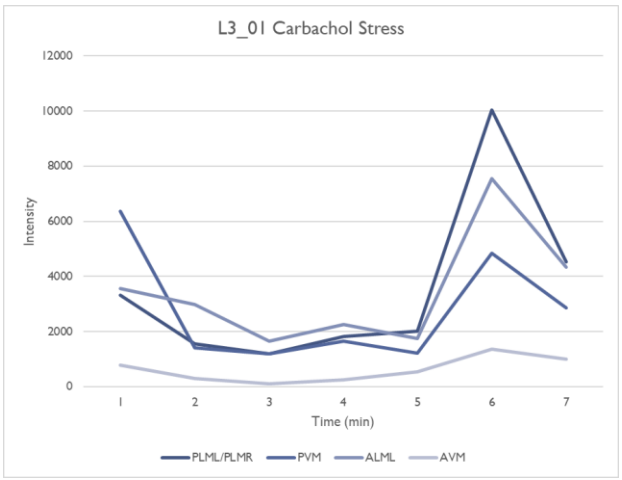
L3_01
L3_02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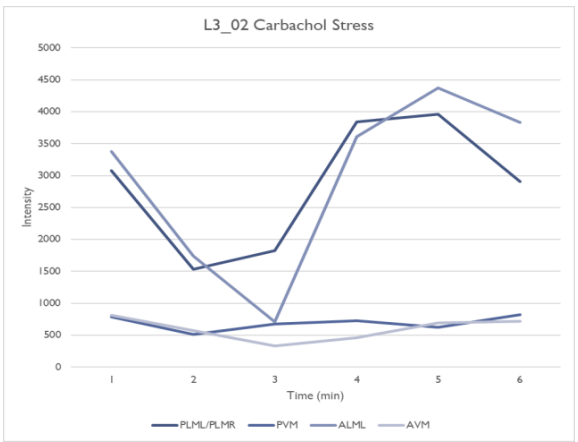
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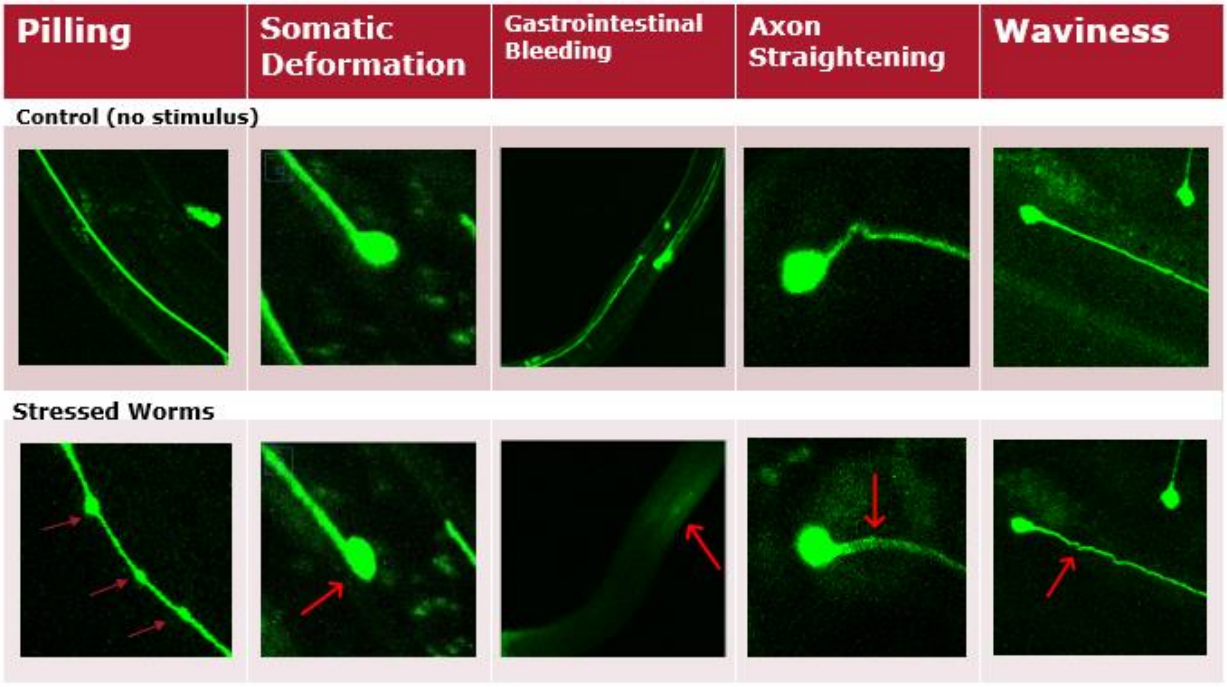
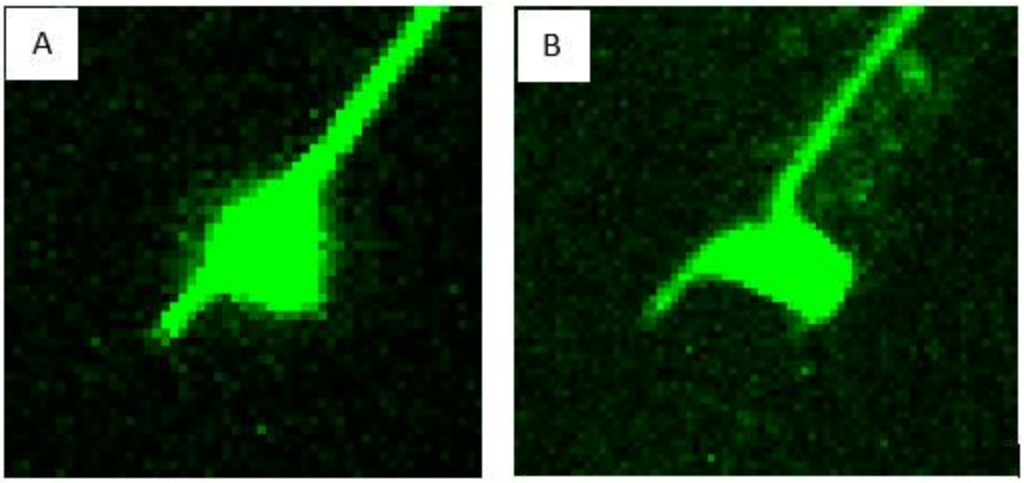
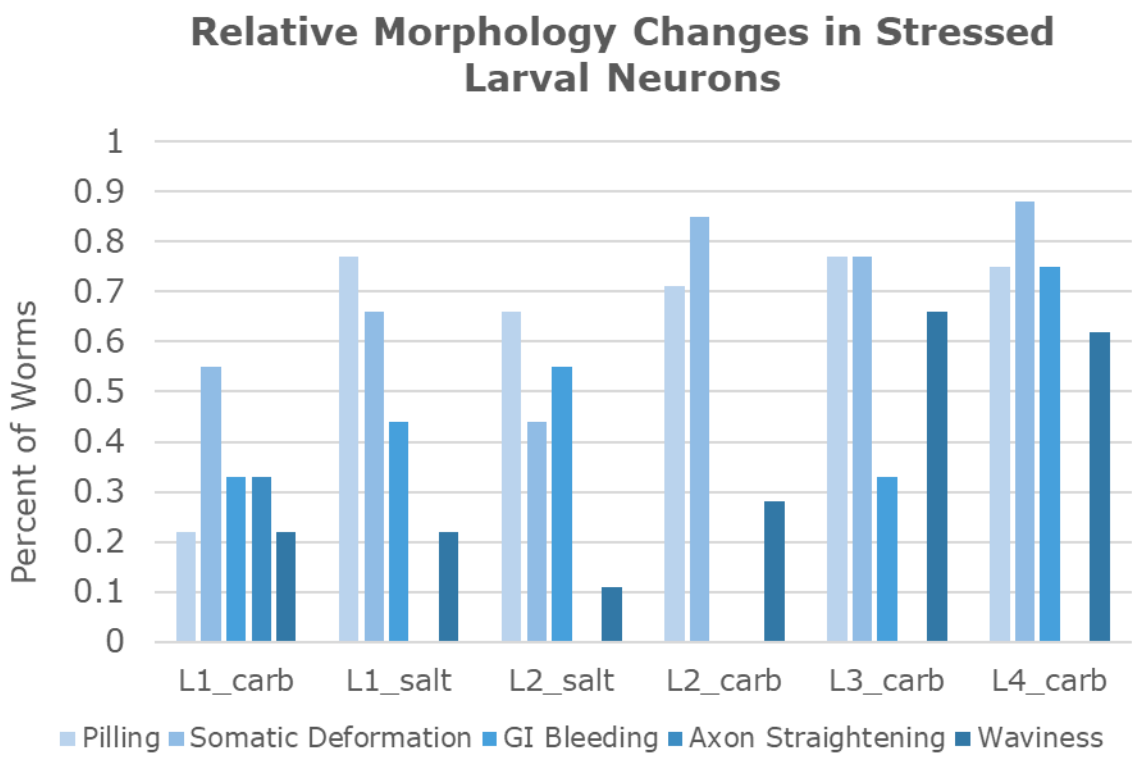

Pilling
Somatic Deformation
Gastrointestinal Bleeding
Axon Straightening
Waviness
Control (no stimulus)
Stressed Worms


A
B


Relative Morphology Changes in Stressed Larval Neurons
Percent of Worms
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
L1_carb
L1_salt
L2_salt
L2_carb
L3_carb
L4_carb
Pilling
Somatic Deformation
GI Bleeding
Axon Straightening
Waviness