THE PENNSYLVANIA STATE UNIVERSITY
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DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

# DYSFUNCTION IN THE BIOSYNTHETIC $NAD^+$ SALVAGE PATHWAY AND PURINE BIOSYNTHESIS PATHWAY ALTER NEUROLOGICAL FUNCTION IN CAENORHABDITIS ELEGANS

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# ABSTRACT

The proper function of specific metabolic pathways is often crucial in normal neuronal functioning. Using the model system *Caenorhabditis elegans*, I investigated the neurological effects of disrupting two metabolic processes: salvage biosynthesis of $NAD^+$ and *de novo* purine biosynthesis.

Nicotinamide (NAM) build-up in *C. elegans* causes death in OLQ neurons, mediated by the OSM-9—OCR-4 TRPV channel. TRPV channels are important mediators of behavioral responses to a variety of exogenous and endogenous stimuli. I investigated another TRPV channel, an OSM-9—OCR-2 TRPV channel known to affect sensitivity to a nose touch response. As knockout of *ocr-4* shows no effect on nose touch response, it is determined that OCR-4 plays no role in this sensory response mechanism. *pnc*-1 knockout mutants, which have increased NAM levels, are shown to exhibit a decreased responsiveness to the nose touch stimulus in more than one condition, indicating that NAM is an agonist for more TRPV channels besides the OSM-9—OCR-4 channel.

ADSL is a crucial enzyme in the purine biosynthesis pathway. When absent in humans, neurological phenotypes, such as seizures and epilepsy, are observed. To test the precise molecular cause of such phenotypes, a neurological phenotype model is necessary. I endeavored to utilize an assay testing gustatory plasticity, or associative learning ability, in the building of such a model. The primary results of this assay are promising, showing that *adsl-1* mutant animals possess an increased ability to learn to avoid previously positive stimuli after a negative experience, as compared to wild-type worms.

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# ACKNOWLEDGEMENTS

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# Chapter 1 : Introduction

## Proper Functioning of Specific Metabolic Processes is Crucial for Normal Neuronal Functioning

The disruption of normal metabolic functioning can have a significant impact on neurological function within organisms. In humans, problems in oxidative energy metabolism and cytochrome *c* oxidase activity have been linked to neurodegenerative diseases such as Alzheimer's, Huntington's, and Parkinson's diseases[1], deficiencies in vitamin E can lead to neuro-muscular difficulties, such as ataxia and areflexia[2], and decreased levels of iron in early development can inhibit metabolic processes in oligodendrocytes, leading to hypomyelination and possibly behavioral changes[3]. By studying and understanding metabolic processes such as these, researchers can develop therapies to circumvent or solve the neurological issues resulting from their dysfunction.

I investigated two different metabolic pathways and their effects on neurological function in the model system *Caenorhabditis elegans*. First, I investigated the salvage biosynthesis pathway of $NAD^+$, to determine the role of NAM, a form of vitamin $B_3$[4], on sensory perception. My second endeavor focused on attempting to create a neurological phenotype model for ADSL-1 deficiency, an enzyme whose absence causes a disruption in the *de novo* purine biosynthesis pathway. In each of my following chapters, I will delve further into the functioning and

importance of these pathways, and describe exactly how their dysfunction can lead to distinct neurological phenotypes.

*C. elegans* is a useful model system to study the relationships I investigated. They are easy to grow and maintain in a laboratory setting, have a relatively quick life cycle, and possess small, yet well-characterized, genomes[5].

## Chapter 2 : Increased Levels of Nicotinamide Lower Responsiveness to Mechanosensory Stimuli

Nicotinamide (NAM), a form of Vitamin $B_3$, is made available to cells in two different ways; it can either be absorbed through the diet or synthesized via the hydrolysis of $NAD^+$[4,6]. Invertebrate cells use NAM to resynthesize $NAD^+$ through the nicotinamidase enzyme PNC-1[4] (Figure 1). When *pnc-1* is knocked out in *C. elegans*, an increase in NAM levels can be seen, up to 10-fold[7]. This spike in NAM levels leads to the death of two different cell types: uterine vulva one (uv1) cells and neural OLQ cells, mechanosensory neurons located in the head of the animal[4,8].

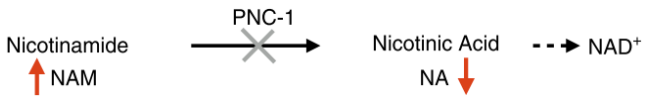



**Figure 1: PNC-1 converts NAM to NA during salvage biosynthesis of $NAD^+$**
Loss of PNC-1 results in accumulation of NAM. Figure and legend borrowed from Upadhyay, et al.[4].

Transient receptor potential (TRP) channels are cation channels, most often found, in *C. elegans*, in the plasma membrane of sensory cells[4,9,10]. TRPV channels are of particular interest,

as they mediate behavioral responses caused by exogenous mechanical, temperature, or chemical stimuli, as well as endogenous modulators[11,12]. Identifying the metabolites that impact these channels is crucial for characterizing the potential links between sensory responses and metabolism[4].

OSM-9 is one of the most abundant TRPV channel proteins in *C. elegans*, and mediates the behavioral response to various mechanosensations[13,14]. OCR subunits, or OSM-9 and capsaicin receptor related subunits, are expressed in cells also expressing OSM-9[13]. OCR-1, OCR-2, and OCR-4 are all expressed in sensory neurons, with OCR-1 being a component of odorant-detecting channels, OCR-4 being a component in channels that process mechanical stimuli, and OCR-2 being a component in channels that process both stimulus types[15].

One notable channel on OLQ cells is the OSM-9—OCR-4 channel, for which NAM is an agonist[4]. An increase in the level of NAM, such as when *pnc-1* is knocked down, leads to a small number of cell deaths among the OLQ neurons[4]. Another TRPV channel in *C. elegans* sensory neurons, the OSM-9—OCR-2 channel, plays an important role in registering the gentle nose touch stimulus[13]. It is unknown whether OCR-4 plays any role in the nose touch stimulus response.

## Materials and Methods

### *Strains and Strain Maintenance*

The following *C. elegans* strains were used: Bristol N2, wild-type; pnc-1(pk9605) IV; CX10 osm-9(ky10) IV; CX4533 ocr-1(ok132) V; LX950 ocr-4(vs137) IV; VM396 ocr-2(ak47) IV.

Animals were fed OP50 *E. coli* and stored at 20$^{o}$C[5]. HT115 *E. coli*, which has an IPTG inducible T7 promoter and is tetracycline resistant, is used in all RNAi experimentation[16]. NGM standard agar was used for most experimentation, except for RNAi experimentation, where it was substituted with specially-made RNAi NGM plates (1mM IPTG, 25µg carbenicillin)[5]. These maintenance procedures were followed for all experimentation in all sections of this thesis.

*Egg Preparation*

Egg preparation (or egg prep) was used to sync all animals to the same stage in their lifecycle. Several healthy plates covered with egg-filled adults were washed with an M9 buffer solution (24mM $Na_2HPO_4$, 11mM $KH_2PO_4$, 4mM NaCl, 9mM $NH_4Cl$, 100µM $MgSO_4$, 5µM $CaCl_2$). The M9-worm solution was then transferred to a tube and gently centrifuged to pellet the worms to the bottom of the tube. The supernatant was then removed and replaced with 400µL bleach and 150µL 3M NaOH, to lyse the adult worms and releases the eggs inside. The worm pellet was swirled in the bleach solution and allowed to sit for 3 minutes. Afterwards, the worms were washed and centrifuged 3 times in M9 buffer. The tube was then filled with 1mL M9 and

allowed to sit on a rocking surface overnight. The next morning, the newly-hatched L1 worms were platted onto new plates seeded with 400µL OP50[18].

*Preparation of Thin-lawn Nose Touch Assay Plates*

NGM plates were spotted with 200µL of an overnight culture of OP50. All plates were made in the same day, then stored at 4ºC to prevent further bacterial growth. The plates were kept for no longer than 10 days. In experiments using RNAi knockdown, RNAi plates were instead spotted with 200µL of an overnight culture of HT115. In assays using dead food, the bacteria were killed by exposure to 125mJ UV radiation for 10 minutes.

*Nose Touch Assay*

An eyelash or eyebrow hair was chosen and sterilized in ethanol. This same hair was used throughout the duration of the assay, so variations in hair width did not cause unintentional discrepancies in animal response. The hair was attached to a toothpick by the root end and placed through a 1000µL pipette tip to provide control and grip.

A single animal was placed on a thin-lawn plate and allowed to roam freely for a minimum of 5 minutes, to desensitize them from the transfer process. The hair was then laid gently on the plate directly in the path of the animal, being careful to hold still and not damage the agar. After the worm's nose hit the hair, three responses were possible (Figure 2). A positive response included a complete reversal or a withdrawal from the obstacle. A negative response

was no reaction to the stimulus. Each worm was touched 10 times, allowing at least 10 seconds between each touch to allow the animal to become desensitized.

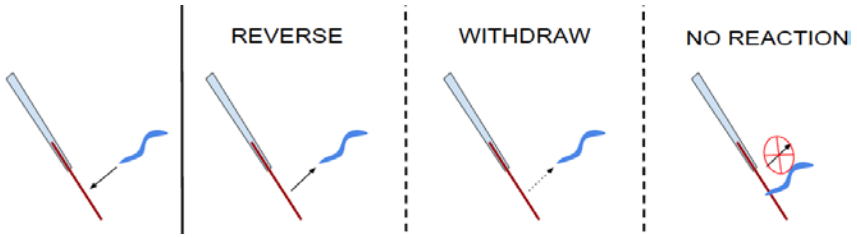



**Figure 2: Nose touch assay method and potential outcomes**
A small hair (attached to a toothpick) was gently laid on the agar in the direct path of the worm. The worm crawled forward until the tip of their nose bumped into the hair. Three reactions were possible. The worm could reverse directions, crawling backwards away from the hair. This was a positive result. The worm could sharply withdraw, but then continue in its initial direction. This was also a positive result. The worm could have no reaction to hitting the hair at all, and try to force its way over, under, or around the obstacle. This was a negative result.

## Results

OSM-9 and OCR-2 are known to be crucial components in a TRPV channel regulating the nose touch response, and therefore nose touch response should be diminished in the absence of these proteins. OCR-1, as it is expressed in chemosensory neurons, should theoretically have no impact on sensing mechanical stimuli. OCR-4 is known to be expressed in mechanosensory cells in the nose of the worm, but it is unknown whether their absence would cause the same change in nose touch response.

To test whether OCR-4 had any effect on the nose touch response, I performed nose touch assays on the following strains: *ocr-1*(ok132) (n=10), *ocr-2*(ak47) (n=10), *ocr-4*(vs137) (n=10), N2 (n=11) and *osm*-9(ky10) (n=10). N2 and *ocr*-1 showed fair response rates of 66% and

56% respectively. The *ocr-2* and *osm*-9 mutant strains exhibited significantly decreased response rates, at 26% and 38% respectively. *ocr-4* responded as the N2 and *ocr-4* strains had, with a response rate of 58% (Figure 3).

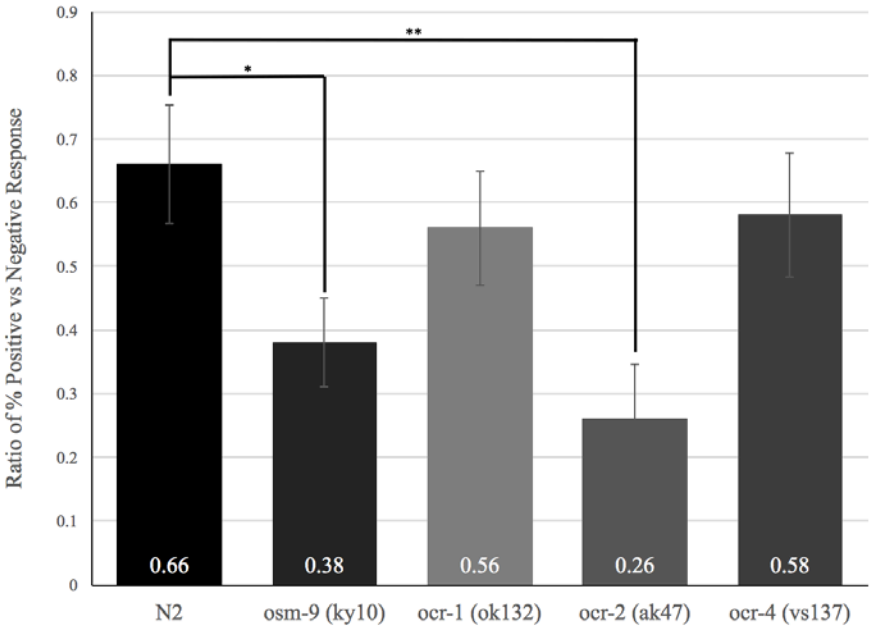



**Figure 3: Nose touch response is inhibited by the knockout of *ocr-2*, but not by *ocr-1* or *ocr-4***
Knocking out *ocr-2* (n=10) decreases sensitivity to the nose touch stimulus more significantly (**p=0.0053, unpaired t-test) even than the *osm-9* mutant (* p=0.0282, unpaired t-test). Knocking out *ocr-1* (n=10) and *ocr-4* (n=10), however, does not significantly decrease sensitivity to the nose touch stimulus.

Nose touch assays were also performed on N2 and *ocr-4* mutants, with and without NAM supplementation. Both N2 (n=18) and *ocr-4* mutants (n=19) had a robust nose touch response rate, 85% and 79% respectively. However, after supplementation with NAM, the responsiveness of the N2 (n=17) and *ocr-4* mutants (n=25) dropped dramatically, to 48% and 46% respectively (Figure 4). The drops in responsiveness of each strain were significant, but not significantly different from each other.

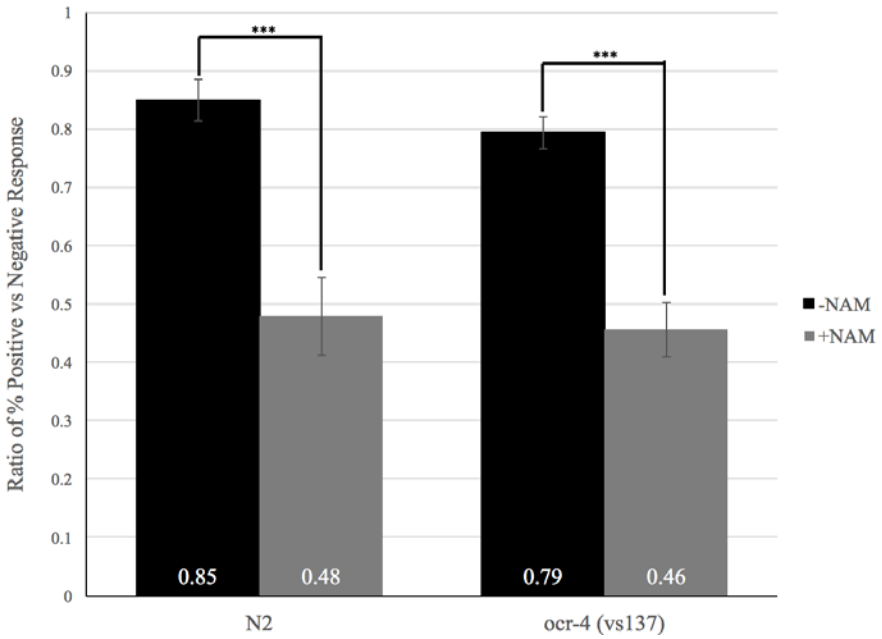



**Figure 4: Supplementation of NAM to *ocr-4* mutants reduced the responsiveness to the nose touch stimuli similar to N2 wild-type**

N2 and *ocr-4* mutant animals showed a similar change responsiveness when supplemented with nicotinamide (NAM). Without NAM, the animals had a high stimulus response rate. When NAM was introduced, the average percent responsiveness was reduced to less than half. The drop in responsiveness between the NAM supplemented and NAM free conditions for both strains is significant, *** $p<0.0001$, unpaired t-test.

Finally, since NAM supplementation had reduced the responsiveness of N2 worms to the nose touch stimulus, *pnc-1* mutants, which are known to have increased cellular levels of NAM, were tested under two conditions: live food and UV-killed dead food. *pnc-1* mutants (n=23) showed a significantly decreased percent nose touch response compared to N2 worms (n=23), 59% compared to 83%, respectively. N2 animals fed dead food (n=28) showed a decreased responsiveness (56%) similar to the nose touch stimuli comparable to the *pnc-1* mutants. *pnc-1* mutants fed a dead food diet (n=17) had a nose touch responsiveness that was lowered even further from the decrease due to the *pnc-1* mutation, at 31% responsiveness (Figure 5).

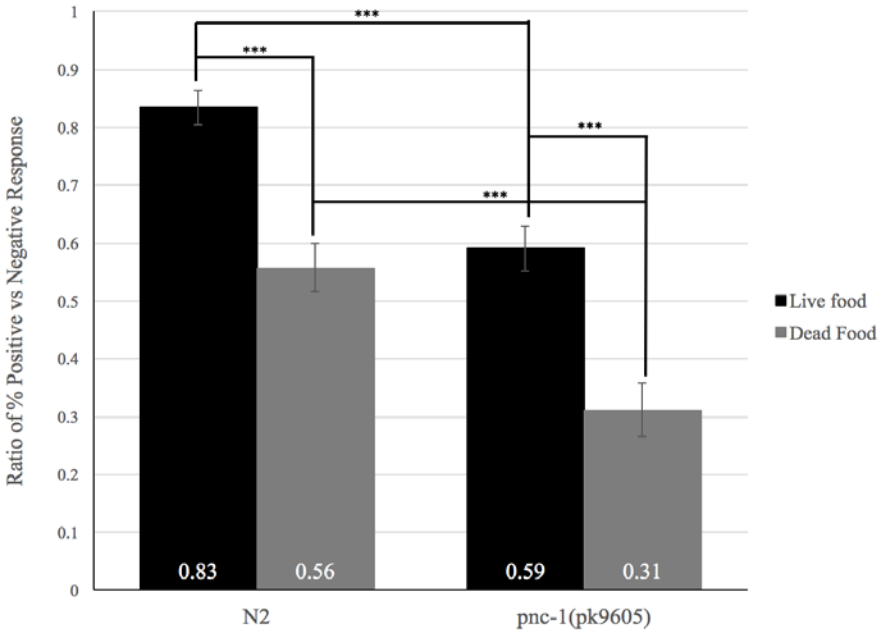



**Figure 5: *pnc-1* mutants have a decreased percentage of nose touch responsiveness compared to wild-type, and a dead food diet decreases the responsiveness of both strains.**
A dead food diet significantly decreases the lifespan of N2 animals, *** $p<0.0001$, unpaired t-test. *pnc-1* RNAi knockout mutants show a decreased sensitivity to the nose touch stimulus when compared to N2 animals, *** $p<0.0001$, unpaired t-test. When *pnc-1* mutants are fed a dead food diet, their responsiveness to the nose touch stimulus decreases a significantly greater amount, *** $p<0.0001$, unpaired t-test. The responsiveness of *pnc-1* mutants fed a dead food diet is significantly lower than the response of N2 fed dead food, *** $p=0.0004$, unpaired t-test.

## Discussion

*ocr-2* and *osm-9* mutants showed a decrease in the nose touch response when compared to wild-type and *ocr-1* worms. This was expected, as these are known components of a channel that regulates response to this stimulus[13], and therefore their absence should lead to a decreased response rate. The lack of a response decrease from the *ocr-1* knockout makes sense, as OCR-1 channels in the nose are most important for odorant sensing, and therefore should not affect how the worm reacts to a mechanical sensation. Since the unknown *ocr-4* mutant responded as the N2

and *ocr-1* strains did, it is implied that OCR-4 subunits play no role in mediating the response to this specific mechanostimulus.

Interestingly, in this assay, the N2 strain did not exhibit nearly the same robust response rate as it has other nose touch assays. This would lead me to doubt the reliability of my results, however other individuals from my lab collected data that displayed a similar distribution of response rates. Therefore, though the response rates in the assay I performed were surprisingly low, the outcome can be trusted.

When N2 worms and *ocr-4* mutants were supplemented with NAM, the nose touch responsiveness rate dropped from nearly identical response rates to nearly identical response rates. This further indicates that *ocr-4* plays no regulatory role in mediating the nose touch response, as knocking out the OCR-4-containing channels had no effect on the observed phenotype. However, it was interesting to see that increased NAM levels altered how the wild-type worms responded to the nose touch stimulus.

*pnc-1* mutants, known to have increased cellular NAM, showed a similar decreased sensitivity to the nose touch stimuli under two feeding conditions: live and dead food. This is an interesting discovery, as we now know that OCR-4 does not affect nose touch, but NAM appears to. The results from Figures 4 and 5 indicates that NAM is not only an agonist for the OSM-9—OCR-4 TRPV channel, but other OLQ cell TRPV channels as well. Further investigating the role NAM plays in these other TRPV channels would be an interesting direction for further study.

# Chapter 3 : Development of Chemotaxis Assay to Model Neurological Phenotype of ADSL Deficiency

Adenylosuccinate lyase (ADSL) is a crucial enzyme in the *de novo* purine biosynthesis pathway[19]. It acts at two different points in the pathway, though the first point of action is of particular interest. Here, ADSL catalyzes the cleavage of succinyl groups from phosphoribosylsuccinyl-aminoimidazole carboxamide (SAICAR) to form phosphoribosylaminoimidazole carboxamide (AICAR) (Figure 6)[20]. In the absence of a ADSL, SAICAR is dephosphorylated to succinylaminoimidazolecarboxamide riboside (SAICAr)[20]. This byproduct accumulates, and becomes detectable in the urine, plasma, and cerebral fluid of individuals with this deficiency[19,21].

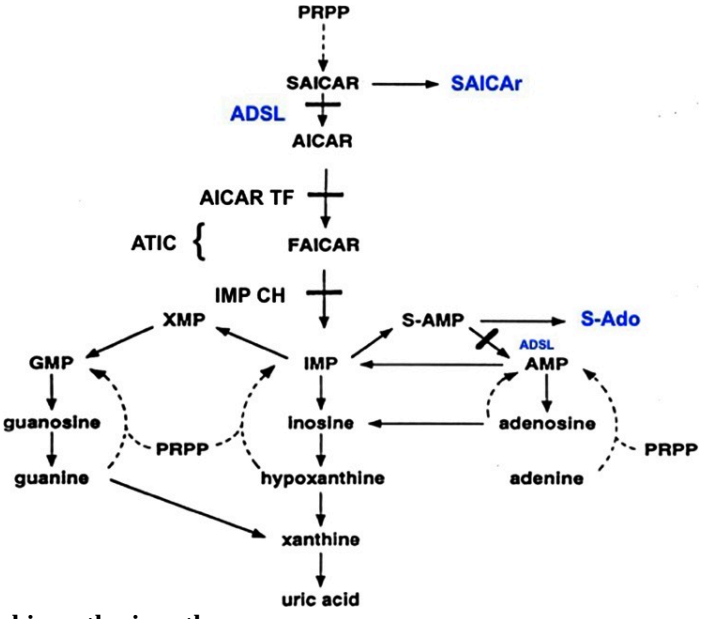



**Figure 6: *de novo* purine biosynthesis pathway**
Both points of action for the Adenylosuccinate lyase (ADSL) enzyme, as well as the respective byproducts of these processes, are shown in blue. Figure borrowed from Jurecka, et al.[20].

ADSL deficiency in humans presents in a number of ways. The deficiency itself is often discovered during childhood, as these children are severely developmentally delayed[22]. Individuals with this disorder express additional phenotypes such as epilepsy, hypotonia, aggressiveness, psychomotor retardation (PMR), muscular wasting, and ataxia[19,21,23,24]. The severity of this disorder can range from neonatal lethal to causing mild autistic symptoms[21].

Since its discovery and characterization in 1984, only around 80 patients have been diagnosed with ADSL deficiency disorder[19,21–23]. As such, ADSL deficiency remains a widely under-researched and under-understood phenomenon.

At the conception of this project, there was one leading question: what is the main cause of the phenotypes of ADSL deficiency disorder? One possibility was that an inability to create purines *de novo* led to a purine shortage, which caused the disorder. The other possibility was that the buildup of SAICAr was toxic. This possibility was supported by the significant amounts of SAICAr that could be detected in the fluids of affected patients. It had been suggested, though never conclusively stated, that varying levels of SAICAr corresponded to the severity of disorder[21,23]. Before either of these theories could be tested, however, a phenotypic disease model was needed.

I attempted to develop an assay model by which to test a neurological phenotype of *C. elegans* with an ADSL deficiency. The idea was to assess the gustatory plasticity of *adsl-1* mutant worms. Gustatory plasticity is the ability of the animals to associate a certain stimulus with a learned negative cue[25]. Wild-type *C. elegans* are attracted to moderate salt concentrations in their environment, as salt is often an indicator of the presence of food[25]. However, after exposure to a high salt concentration under starvation conditions, wild-type worms will learn to avoid salt in their environment[26,27].

In humans, clear neurological phenotypes can be observed in ADSL-deficient individuals, particularly developmental delays. Should a *C. elegans* model be established that shows a learning delay in *adsl-1* worms, rescue experiments could be run in an attempt to pinpoint the specific biological cause of the phenotype.

## Materials and Methods

### *Plate and Buffer Preparation*

CTX buffers, containing 5mM $KHPO_4$, 1mM $CaCl_2$, and 1mM $MgSO_4$, were prepared, containing either 100mM NaCl or no salt. 1.7% agar solutions were also prepared, containing the salt-less CTX buffer and either 25mM NaCl or no salt. Agar was poured into each of the four quadrants of a Falcon X plate, with CTX control agar in diagonal quadrants, and CTX salt agar in the other two quadrants (Figure 7A). The plastic ridges between the quadrants were coated in a thin layer of CTX control agar to connect all quadrants together.

### *RNA interference (RNAi) by feeding*

RNAi plates were seeded with HT115, transformed with a dsRNA plasmid containing a sequence homologous to that of the target worm gene. N2 worms were washed with M9 to remove any OP50 from their previous plate, and plated on the RNAi plates. The animals consumed the HT115 and the dsRNA plasmid was absorbed through the gut[17].

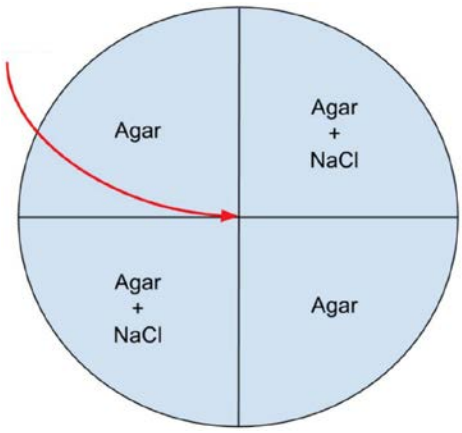
### *Strain Preparation*

The Bristol N2, wild-type *C. elegans* strain was used during this experimentation. The worm strains were egg prepped and allowed to age for 3 days on a lawn of 400µL HT115. Worms from each strain were then separated into two groups: some plates were washed in CTX control buffer and some were washed in CTX salt buffer. The strains were rocked for 15 minutes in their respective buffer washes. Afterwards, the tubes were gently centrifuged and the buffer was removed (Figure 7B).

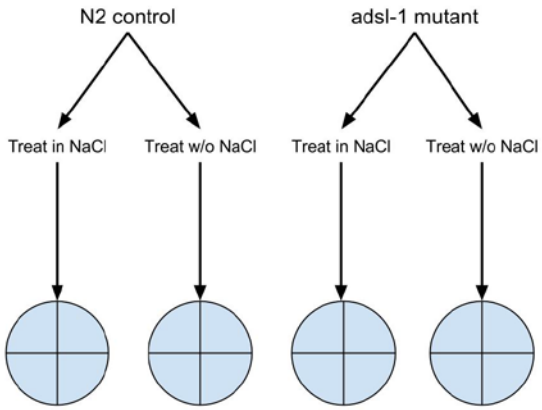
*Chemotaxis Assay*

Animals were placed in the center of the four quadrant plate using a glass pipette (Figure 7A,B). Any remaining liquid was immediately removed from the plate with a kimwipe. The worms were then left to crawl around the plate for 30 minutes. After 30 minutes, the number of worms present in each quadrant were counted and the chemotaxis index was calculated (Figure 7C).

**A**




**B**



**C**

$$Chemotaxis\ Index\ (CI) = \frac{\#\ worms\ on\ NaCl\ quadrants - \#\ worms\ on\ control\ quadrants}{Total\ \#\ of\ worms\ on\ plate}$$

**Figure 7: Preparation and analysis of chemotaxis assay to test gustatory plasticity**
**(A)** Each strain was tested under two conditions. One condition treated the worms in a high concentration of salt (100mM) for 30 minutes, directly prior to plating. The other condition took the worms through the same treatment process, but did not expose them to high concentrations of salt. Both conditions of both strains were then plated on separate four-quadrant plates. **(B)** The plate on which this assay was run is split into four equal quadrants. Two diagonal quadrants contained 25mM NaCl CTX. The remaining two quadrants contained CTX agar with no salt concentration (control). The plastic walls separating the quadrants were coated in a thin layer of the control agar and the worms were placed directly in the center of the plate (red arrow). **(C)** Gustatory plasticity was measured using a chemotaxis index (CI) for each condition. Chemotaxis index was calculated by subtracting the number of worms in the control agar quadrants from the number of worms in the salt agar quadrants, then dividing by the total number of worms plated.

## Results

Both N2 worms and RNAi *adsl-1* mutants were pretreated in NaCl or salt-free buffer and tested using this proposed assay method, using the control and RNAi-modified strains. Five trials were run over the course of one month. All of these trials were completed on separate days, apart from the final two trials, which were completed in tandem with one another.

Naïve worms from both the wild-type and experimental strains were heavily attracted to the salt-containing agar segments, consistent with previously published data. The naïve empty vector-containing strain expressed an average chemotaxis index of 0.817 (SD=0.1005), and the naïve adsl-1 RNAi knockdown strain had an average chemotaxis index of 0.853 (SD=0.0780) (Figure 8). This result demonstrates that the ability of the *adsl-1* mutants to chemosense is equal to that of the N2 control strain. The empty vector-containing animals pretreated in 100mM salt buffer preferred neither kind of agar, with a chemotaxis index of -0.380 (SD=0.3276) (Figure 8). Interestingly, the N2 *adsl-1*RNAi knockdown strain, when pretreated with high salt, showed an

aversion to the salt agar quadrants (CI=-0.414, SD=0.2600), indicating it had learned to associate salt with the negative cue better than the wild-type worms (Figure 8).

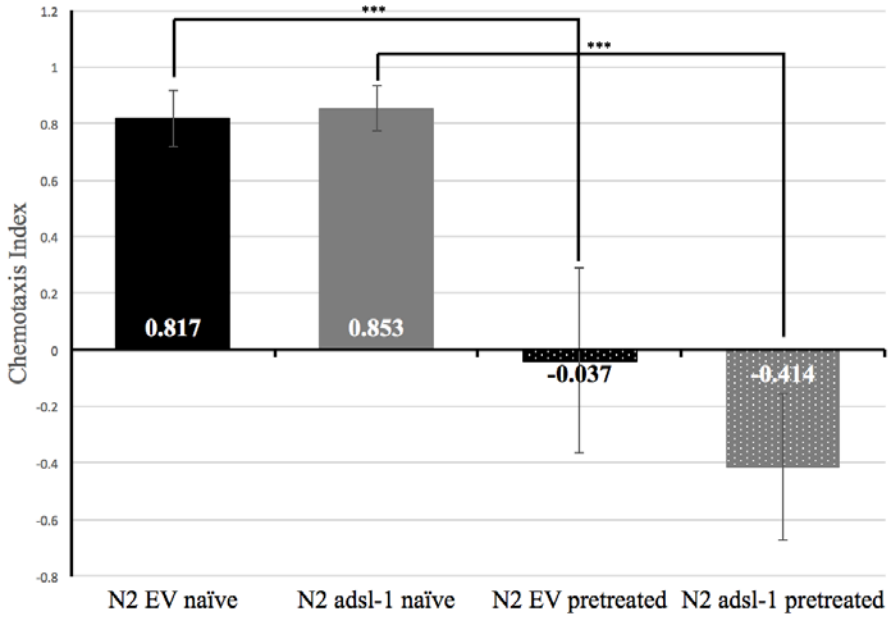



**Figure 8: N2 *adsl-1* knockout mutants show learned aversion towards salt agar, but not the empty vector control**

The chemotaxis index (CI) for each trial condition was calculated and averaged. The N2 EV naïve condition (n = 666 animals over 5 trials) had a CI of 0.817, indicating a preference towards the salt agar quadrants after 30 minutes. The N2 *adsl-1* naïve condition (n = 904 animals over 5 trials) had a CI of 0.853, indicating a preference towards the salt agar quadrants after 30 minutes. When the N2 EV condition was pretreated with 100nM NaCl (n = 294 animals over 5 trials), its CI dropped to -0.037 (*** p=0.0005, unpaired t-test), indicating no preference towards salt or control agar. When the N2 *adsl-1* condition was pretreated with 100mM salt (n = 259 animals over 5 trials), its CI dropped to -0.414 (*** p<0.0001, unpaired t-test), indicating a slight preference for the control agar quadrants after 30 minutes. The difference in agar preference between the N2 EV and N2 *adsl-1* knockdown strains was not quite significant, p=0.0791, unpaired t-test.

## Discussion

As it stands, the data collected from these initial trails shows a promising start towards the creation of a neurological phenotype model for *adsl-1* mutants. The results from both the N2 EV and N2 *adsl-1* naïve conditions were what we had hoped to see out of our control strains;

both were highly preferential towards the salt agar when untreated with high levels of salt. As the chemosensory abilities of both strains were nearly identical, it is possible to attribute any observed discrepancies between the strains after salt pretreatment to a difference in learning ability.

Pretreating the N2 EV condition in 100mM NaCl caused the animals to be indifferent towards the different agar conditions. However, pretreating the N2 *adsl-1* knockdown mutants in the same way caused the animals to noticeably avoid the salt agar. This result indicates that there is an observable neurological phenotype in *adsl-1* mutants: they have increased gustatory plasticity compared to wild-type worms. In my experimentation, though the results are promising, the difference between the pretreated strains is just statistically insignificant; however, this same experimentation has been repeated after me, and these results have been consistent across different experimenters. Therefore, I am confident in saying that we have established a neurological phenotype model for ADSL-1 deficiency.

The fact that the *adsl-1* mutants had an increased ability to associate stimuli with a negative experience is interesting, as the human developmental delay phenotype would have led me to believe the opposite more likely. However, any difference between the wild-type and mutant strains is encouraging, as any phenotypic difference has the potential to be rescued.

The future plan for this model is that it be used to test if blocking the formation of SAICAr from SAICAR rescues the neurological phenotype. In order to do this, I would block the purine biosynthesis pathway above the action of ADSL, so purines are still unable to be created *de novo*, but SAICAr is unable to be formed. If blocking the pathway higher up rescues the learning phenotype, then that would support the hypothesis that the buildup of SAICAr is the primary cause of the neurological defect. Any other result, either no change in the gustatory

plasticity of the worms, or an increased avoidance of the salt agar, would not support this hypothesis, and therefore other potential causes of the neurological abnormalities would have to be investigated.

## Appendix A : The Effects of Pyruvate Carboxylase on the Extended Lifespan of Mitochondrial Sirtuin Mutants is Unclear

Sirtuins are a group of $NAD^+$-dependent protein deacetylases, whose main functions are to regulate metabolism, development, longevity, and stress response[28]. *C. elegans* have four sirtuins, SIR-2.1 – SIR-2.4, with SIR-2.2 and SIR-2.3 being mitochondrial[29,30]. Humans have 7 sirtuins, with SIRT3, SIRT4 and SIRT5 being mitochondrial, and SIRT4 being an ortholog of the *C. elegans* mitochondrial sirtuins[30,31].

Studies have linked dysfunction of mitochondrial genes to a several age- and metabolism-related diseases, such as cancer[32], diabetes[33], metabolic syndrome[34], and neurodegenerative diseases, like Alzheimer's or Huntington's disease[35]. As one of their primary roles is to regulate metabolism, mitochondrial sirtuins could be used as therapeutic targets in trying to combat such diseases[34]. However, before this is a possibility, mitochondrial sirtuins need to be further studied and understood.

Overexpression of nuclear SIR-2.1 is known to increase average lifespan of *C. elegans* by 50%[36]. Interestingly, mitochondrial sirtuin knockout mutants *sir-2.2*(tm2673) and *sir-2.3*(ok444) also exhibit an extended lifespan phenotype when fed the typical *E. coli* OP50 diet[37]. *sir-2.2* mutant animals live an average of 4 days longer than N2 wild type animals, while *sir-2.3* mutants live an average of 3.6 days longer (Figure 9). The exact metabolic processes that cause this lifespan increase, however, have yet to be characterized.

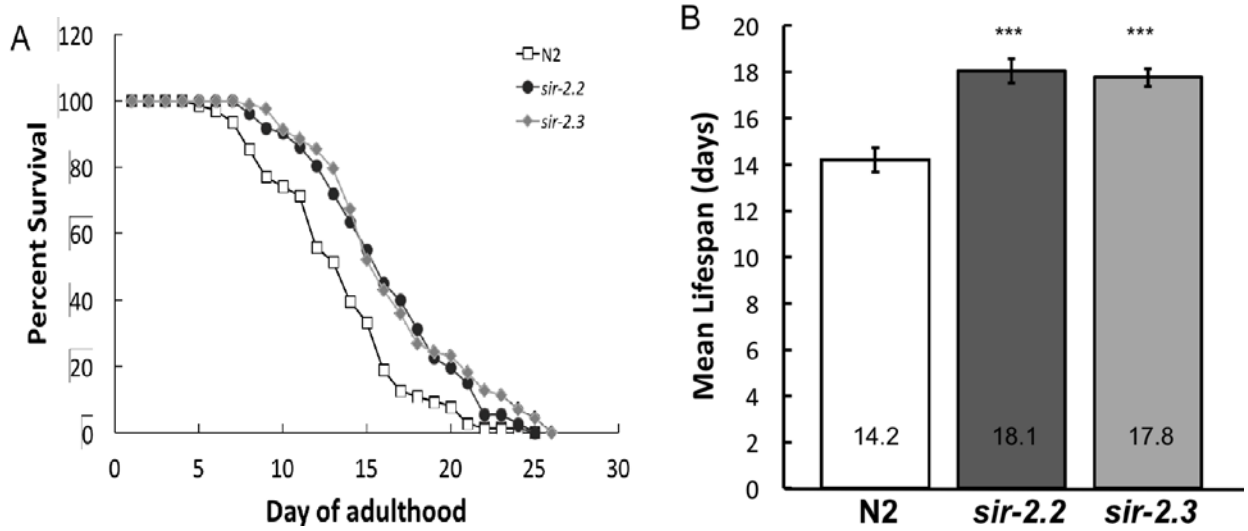



**Figure 9: sir-2.2(tm2673) and sir-2.3(ok444) live longer than N2 wild-type when fed ad libitum**
**(A)** Survival curve displaying values from the average of three independent experiments, $p<0.001$, log rank t-test. **(B)** Mean lifespan values from twelve independent experiments ±SEM, ***$p<0.001$, unpaired t-test. Figure and legend borrowed from Chang[37].

Previous attempts at running RNAi experimentation on *sir-2.2* and *sir-2.3* mutants have brought to light a puzzling issue. Mitochondrial sirtuin mutants fed the RNAi vector, HT115, no longer exhibit the same lifespan extension phenotype that they did when fed OP50; in fact, the mutants no longer show any lifespan extension over N2 at all (Figure 10). While experimentation using RNAi still continued, it became necessary to think of ways to circumvent this issue, and this issue was a factor in analyzing any data gathered.

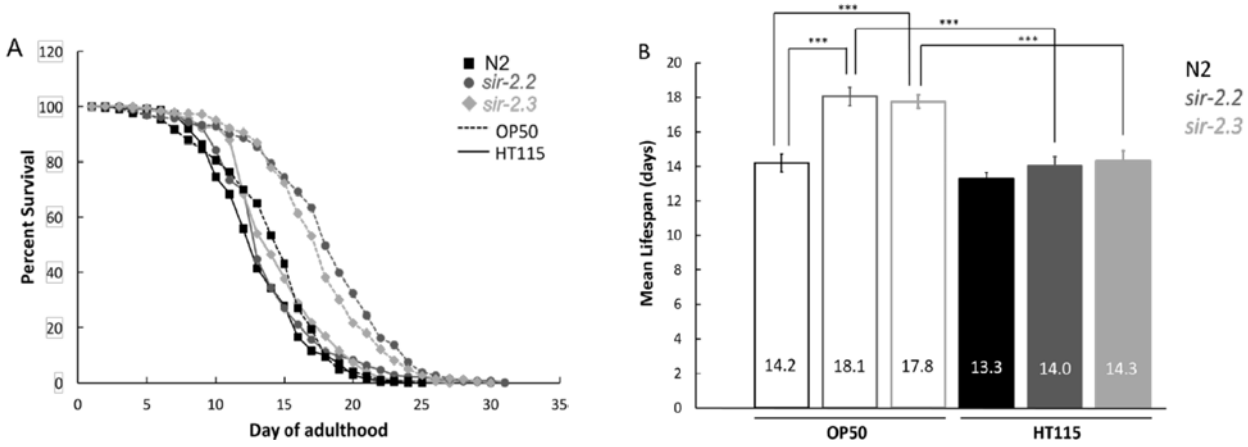



**Figure 10: sir-2.2 and sir-2.3 extended lifespan on OP50 is no longer observed when fed HT115**

Lifespans of N2, sir-2.2, and sir-2.3 were measured on OP50 and compared with animals fed HT115. **(A)** Survival curves for N2, sir-2.2, and sir-2.3 on either OP50 or HT115. OP50 values are the average of twelve independent experiments and HT115 values are the average of nine independent experiments. There is a significant difference between sir-2.2 and sir-2.3 fed OP50 and sir-2.2 and sir-2.3 fed HT115, $p<0.001$ log rank t-test. **(B)** Mean lifespan of animals on OP50 and HT115 ±SEM, *** $p<0.001$ unpaired t-test. Figure and legend borrowed from Chang[37].

SIR-2.2 and SIR-2.3 are known to interact with mitochondrial biotin-dependent carboxylases, as is the human ortholog SIRT4[30]. Some of these include pyruvate carboxylase (*pyc-1),* propionyl-coenzyme A (-CoA) carboxylase (*pcca-1*), and methylcrotonoyl-coenzyme A (-CoA) carboxylase (*mccc-1*)[30]. Pyruvate carboxylase in particular functions in the TCA cycle, in the ATP-dependent process of converting pyruvate to oxaloacetate[38,39]. Though it is known that these carboxylases and sirtuins interact, how their interactions contribute to the sirtuins regulation of lifespan remains unknown.

## Materials and Methods

### *Strains*

The following *C. elegans* strains were used: Bristol N2, wild-type; RB654 sir-2.3(ok444); sir-2.2(tm2673).

### *Lifespan Assay*

An egg prep was performed and the L1 larva were plated on an RNAi plate seeded with 400µL of HT115 containing the desired plasmid. The HT115 came from a liquid culture containing LB broth, 50µg/ml ampicillin, and 12µg/ml tetracycline. After two days, or when the worms had grown to L4 larva, approximately 90 worms were picked and placed on 3 plates (approximately 30 worms to a plate). The number of dead worms on each plate each day were

recorded. The live worms were transferred onto new 400µL HT115 plates daily, so as not to confuse the original animals with their progeny. Deceased worms who had bagged (eggs hatched inside the mother) or exhibited the "exploded vulva" phenotype were not counted towards the death count. This process continued until all of the worms on each plate had died. The results represent an average of all 3 plates per strain (Figure 11). All experiments were done on the bench at 20$^{o}$C.

## Results

In order to investigate the possible effects pyruvate carboxylase might have on lifespan, assays examining the average lifespan of N2 worms, *sir-2.2* mutants, and *sir-2.3* mutants with *pyc-1* knocked down via RNAi were run. These assays were run at separate times, but were identical in procedure. In the initial assay, *pyc-1* knockdown seemed to increase the lifespan of both *sir-2.2* and *sir-2.3* mutants, but neither result was deemed statistically significant using an unpaired t-test (Figure 11A). In the second trial, all of the *pyc-1* knockdown mutants showed decreases in lifespan extension, and the decrease in the *sir-2.3* mutant lifespan was significant (Figure 11B).

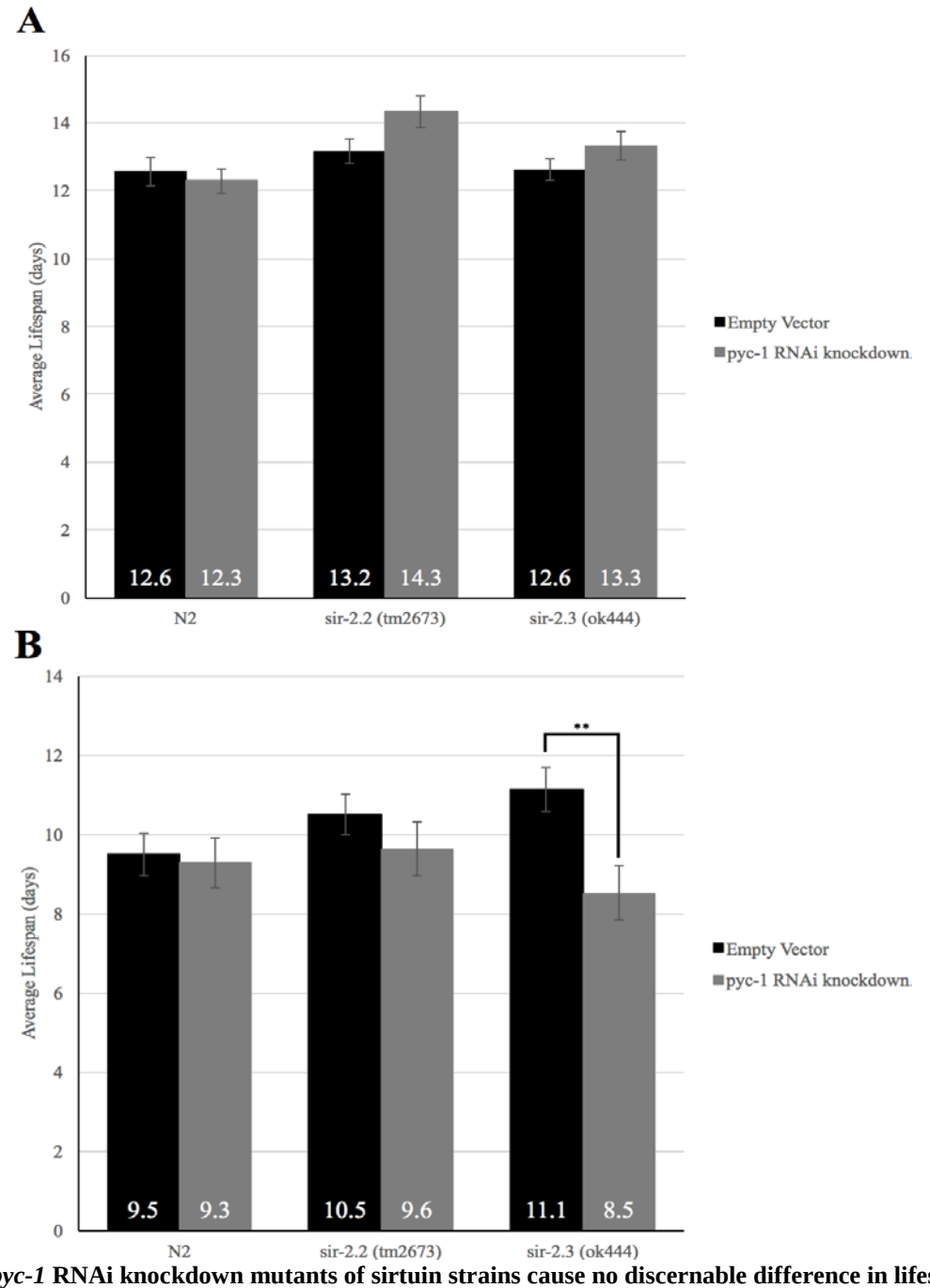



**Figure 11: *pyc-1* RNAi knockdown mutants of sirtuin strains cause no discernable difference in lifespan**
2 trials were run testing the lifespan of *sir-2.2* and *sir-2.3* when pyc-1 was knocked down using RNAi. **(A)** Knocking down *pyc-1* increased the lifespan of both sirtuin mutants, but neither increase was significant (p=0.0560, unpaired t-test) **(B)** During the second trial, both sirtuin mutants had decreased lifespans when *pyc-1* was knocked down. The lifespan decrease seen by the *pyc-1* knockdown in sir-2.3 was significant, ** p=0.0033, unpaired t-test.

## Discussion

The effect of pyruvate carboxylase on the extended lifespan of mitochondrial sirtuin mutants is still unclear. In the first assay trial, there appeared to be an increase in the lifespan of *sir-2.2* and *sir-2.3* mutants when *pyc-1* was knocked down, albeit these results barely fell short of significant. Ideally, as the original n was lower than desired, a repeat of the experiment would solidify whether or not the result was significant. While the second trial supported the insignificance of the lifespan increase, it added a myriad of additional questions. In the second run of the experiment, the knockdown of *pyc-1* decreased the average lifespan of the sirtuin mutants, significantly for *sir-2.3*. Two completely different outcomes resulting from the same experimentation method has led me to distrust any of my results and causes me to think that a repetition of this experiment may be a waste of time.

It is important to note, however, that in the second trial, while the knockdown of *pyc-1* in the *sir-2.2* and *sir-2.3* mutants decreased their lifespan, the lifespan of all the strains were shorter than seen in previous experimentation. N2 animals in particular only lived an average of 9.5 days, where their typical lifespan has proven to be between 12-14 days. This leads me to believe that perhaps other factors were at play that led to the decrease in lifespan for *pyc-1* knockdown mutants in the second trial, such as additional unseen levels of stress. Therefore, looking into the effects of *pyc-1* on mitochondrial sirtuins may still be a worthwhile endeavor, but not by using the same method.

The bulk of this lack of clarity, however, most likely stems from the ineffectiveness of the current RNAi method as a tool to analyze the lifespan extension of sirtuin mutants. Because the lifespans of the sirtuin mutants were not significantly increased on a diet of HT115, as they

are known to be when fed a diet of OP50, there are other methods that would be more effective at investigating the role of PYC-1 in lifespan. One such method would be the use of a mutant worm strain that has a deletion in the *pyc-1* gene. This method would be more time-consuming, and include crossing the *pyc-1* mutant and *sir-2.2* or *sir-2.3* mutant strains, but would hopefully produce more clear and credible results. In addition, other methods of RNAi, such as RNAi by injection, could possibly be utilized.

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ACADEMIC VITA

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## Education

Pennsylvania State University Aug 2014 – May 2018

B.S. in Microbiology, German Minor
Schreyer Honors College
Dean's List: Fall 2014, Spring 2015, Fall 2016

## Work Experience

Laboratory Work: Hanna-Rose Lab at the Pennsylvania State University Jan 2015 – May 2017

- Contributed to projects on Molecular Genetics of Metabolism and Development in *C. elegans,* leading to senior honors thesis
- Presented research at multiple Penn State poster presentations
- Published: Upadhyay, A., Pisupati, A., Jegla, T., Crook, M., Mickolajczyk, K. J., Shorey, M., **Rohan, L. E.**, Billings, K. A., Rolls, M. M., Hancock, W. O., Hanna-Rose, W. (2016). Vitamin B3 / Nicotinamide is an endogenous agonist for a C. elegans TRPV OSM-9/OCR-4 channel. *Nature Communications*, 7: 1–11.

## Skills

*Technical Experience*: Analysis of mass spectral data, Analysis of 1H and 13C NMR, Bacterial culture identification, Bacterial transformation, *C. elegans* behavioral assays, Crystallization, Distillation, DNA isolation, Gas chromatography, Gel electrophoresis, Bacteriophage isolation, IR spectroscopy, Light microscopy, Medium preparation, PCR, Recombinant DNA Techniques, Introductory Spectrophotometry, Simple organic synthesis

*Computer Skills*: Microsoft EXCEL, Microsoft PowerPoint, Microsoft Word, Minitab, NIS-Elements Microsoft Imaging Software

*Language Skills*: Conversational-level German

## Volunteer Experiences

Taproot Kitchen Non-Profit Volunteer Fall 2014 – Present

- Volunteered at events connecting young adults with disabilities to their local communities

Certified PA Literacy Corps Volunteer Jan 2017 – April 2017

- Taught twice-weekly tutoring session to adult ESL learner

Special Olympics Unified Team Partner Fall 2011 – Spring 2013

- Partnered with autistic sister and other special needs athletes to promote meaningful inclusion

## Miscellaneous Experiences

Eberly College of Science Tour Guide Oct 2015 – Present

- Created a friendly and welcoming environment for prospective Penn State science students

Penn State Swing Dance Club Executive Board Fall 2014 – Present

- Planned twice-weekly dance lessons and larger intercollegiate events for 200+ people

Pennsylvania State University Study Abroad: Shanghai, China May 2015 – June 2015

- Honed cross-cultural communication skills with students from different backgrounds