IMPACT OF VITAMIN B12 ON *C. ELEGANS* MODELS OF ALZHEIMER'S DISEASE

by

John Salsini-Tobias

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Honors Degree in Biological Sciences with Distinction.

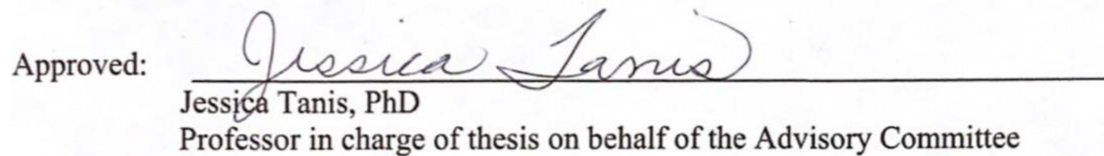
Spring 2023

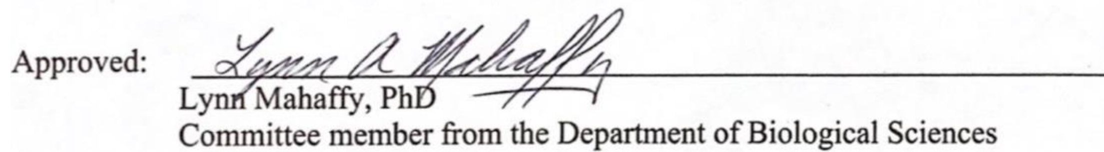
© 2023 John Salsini-Tobias
All Rights Reserved

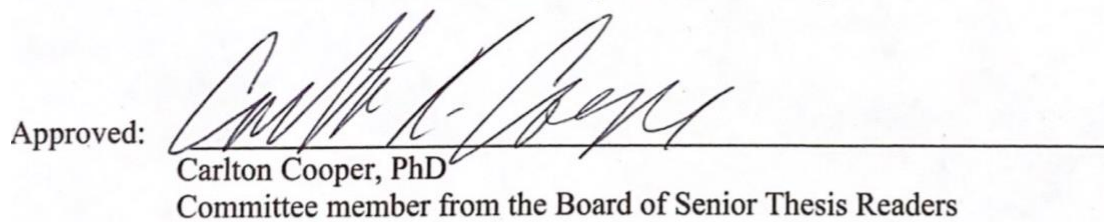
IMPACT OF VITAMIN B12 ON *C. ELEGANS* MODELS OF ALZHEIMER'S DISEASE

by

John Salsini-Tobias


Approved: ______________________
Jessica Tanis, PhD
Professor in charge of thesis on behalf of the Advisory Committee


Approved: ______________________
Lynn Mahaffy, PhD
Committee member from the Department of Biological Sciences


Approved: ______________________
Carlton Cooper, PhD
Committee member from the Board of Senior Thesis Readers

Approved: ______________________
Michael Chajes, PhD
Dean, University Honors Program

ACKNOWLEDGMENTS

When I sent my first email to Dr. Jessica Tanis to ask to join her laboratory, I didn't expect that the time would fly by this quickly. I owe so much to Dr. Tanis's mentorship and guidance both in and out of the lab. From PI to advisor, she has helped me with simple lab experiments and the complicated road to medical school; I would not be seeing the scientific and personal successes I do without her. Of course, my fellow lab members are indispensable in their help during lab work and they create a wonderful sense of belonging in the Tanis lab (the best lab). I am confident that this group of researchers will continue to assist new generations of undergrads in becoming able and knowledgeable. I'd like to specifically thank Dr. Andy Lam for always having time to answer my omnipresent questions, even as he transitioned from graduate student to post-doctoral researcher. Rachel Wang, Alison Davis, Charlotte Leslie, Karli Sunnergren, and Alexis Semmel also deserve a special shout-out for being great helps on the amyloid-beta project and for helping me get started with new protocols and techniques. To the current lab members with whom I haven't had the pleasure to work closely – Malek Elsayyid, Emylee Kerslake, Nahin Siara Prova, Vasilios Lomis, Carter O'Brien, Aarushii Taneja, Zachary Machiorlete, McKenna Millar, and Krisha Parekh – I'm so glad to have you contribute to the great lab atmosphere. I'd like to thank the Undergraduate Research Program and the Honors College for funding me outside of the spring and fall semesters. Thank you to my committee – Dr. Tanis, Dr. Lynn Mahaffy, Dr. Carlton Cooper – for all your time and encouragement. Finally, I'd like to thank my friends for giving me things to do outside of lab work, and my family for fostering my love of science and of writing.

TABLE OF CONTENTS

ACKNOWLEDGMENTS iii
LIST OF TABLES v
LIST OF FIGURES vi
ABSTRACT vii

1 Introduction 1

1.1 Alzheimer's Disease 1
1.2 *Caenorhabditis elegans* 3
1.3 Amyloid-beta Transgenes 4
1.4 Diet in Alzheimer's Disease and in *C. elegans* 5
1.5 SAM/Methionine Cycle 6
1.6 Fatty Acid Synthesis 8
1.7 Objectives and Hypotheses 9

2 Methods 11

2.1 *C. elegans* Maintenance 11
2.2 Supplementing NGM Plates 13
2.3 Genetic Crossing and Genotyping of *C. elegans* 13
2.4 Paralysis Assay 14
2.5 Chemotaxis and Conditioning Assays 15

3 Results 20

3.1 Genetic Crosses 20
3.2 The *fat-7* mutation does not impact Aβ-induced paralysis 22
3.3 The *fat-5* mutation does not impact Aβ-induced paralysis 23
3.4 Chemotaxis Assay with Choline and Vitamin B12 Supplementation 26
3.5 Conditioning Assay 29

4 Discussion 34

REFERENCES 41

LIST OF TABLES

TABLE 1: *C. ELEGANS* STRAINS 12

TABLE 2: PRIMERS FOR GENOTYPING 14

LIST OF FIGURES

FIGURE 1: AMYLOID PRECURSOR PROTEIN CLEAVAGE PATHWAYS. 2

FIGURE 2: PHOSPHOLIPID SYNTHESIS AND THE METHIONINE/SAM CYCLE............. 7

FIGURE 3: FATTY ACID SYNTHESIS IN *C. ELEGANS*.. 9

FIGURE 4: ASSAY TEST PLATE LAYOUT. 17

FIGURE 5: CHEMOTAXIS INDEX EQUATION.. 19

FIGURE 6: GENETIC CROSS SCHEMATIC BETWEEN *FAT-7* POINT MUTANT AND *DVIS100* TRANSGENE. 21

FIGURE 7: GEL IMAGE FROM SUCCESSFUL CROSS OF *FAT-7* AND *DVIS100*. 22

FIGURE 8: *FAT-7* DELETION HAD NO EFFECT ON MEDIAN PARALYSIS. 23

FIGURE 9: ISOLATION OF A NEW *FAT-5* DELETION MUTANT. 25

FIGURE 10: AB-INDUCED PARALYSIS IN WILD-TYPE AND *FAT-5* MUTANT ANIMALS. 26

FIGURE 11: GENETIC CROSS SCHEMATIC BETWEEN *SPE-9* MUTANT AND *GNAIS1* OR *GNAIS2* TRANSGENE.. 28

FIGURE 12: CHEMOTAXIS ASSAYS WITH B12 AND CHOLINE SUPPLEMENTATION.29

FIGURE 13: CHEMOTAXIS INDEX FOR ANIMALS EXPOSED TO AVERSIVE CONDITIONING. 30

FIGURE 14: INDIVIDUAL TRIAL RESULTS OF CONDITIONING DATA......................... 32

FIGURE 15: CONTROL STRAIN CONDITIONING DATA. 33

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by atrophy of neurons in the brain and there are currently an estimated 6.5 million individuals afflicted in the United States. Evidence suggests that build-up of toxic amyloid-beta (Aβ) in the brain is a pathogenic feature of AD. The genetic model organism *C. elegans* provides a useful system to examine how the modifiable risk factor diet affects Aβ-induced proteotoxicity. Expression of Aβ in *C. elegans* body-wall muscles causes time-dependent paralysis, allowing for easy determination of factors that impact proteotoxicity. Previously, the Tanis lab has shown that supplementing with the nutrients vitamin B12 and choline protects against Aβ induced paralysis and bioenergetic defects by impacting the methionine/SAM cycle. Phosphatidylcholine (PtdCho) has been observed at reduced levels in individuals with AD and can be synthesized by SAM-dependent methylation of phosphoethanolamine or directly from choline through the Kennedy Pathway. To further explore the protective potential of vitamin B12 and choline, I employed a *C. elegans* strain that expresses Aβ pan-neuronally and exhibits chemotaxis defects in response to the attractant isoamyl alcohol (IA). Attraction to IA was quantified with a chemotaxis index (CI). In this assay, all groups were attracted to the IA treatment, with a significantly lesser attraction observed for Aβ-expressing animals without B12 supplementation. This suggests that Aβ was detrimental to chemotaxis ability and that vitamin B12 supplementation, but not choline supplementation, is protective against Aβ-induced proteotoxicity in this neuronal model. I then developed a protocol for conditioning the animals to IA in the absence of food before performing the assay to determine if the animals could learn that IA is associated with starvation, however, the results were inconsistent.

Another area of my thesis work focused on the impact of fatty acid synthesis on Aβ-proteotoxicity. Vitamin B12 availability in the diet decreases transcription of the desaturases

FAT-5 and FAT-7. To determine if loss of *fat-7* affected the protective impact of vitamin B12, I created a strain with a mutation in *fat-7* and the body-wall muscle Aβ transgene by genetic recombination. In addition, I used CRISPR-Cas9 to knockout *fat-5* in the Aβ animals. We then performed paralysis assays and found that loss of either *fat-5* or *fat-7* did not impact the vitamin B12 dependent delay in paralysis, indicating that these genes are not required for the protective effect of B12. In conclusion, vitamin B12 has a protective effect in multiple *C. elegans* models of Aβ-induced proteotoxicity and this is independent of its impact on fatty acid synthesis.

1. Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by atrophy of neurons in the brain and has an estimated 6.5 million cases in the United States, which is projected to grow to 12.7 million cases by 2050 as the population ages (Alzheimer's Association, 2022). Notably, AD is the most common cause of dementia (Butterfield & Halliwell, 2019; Chakravorty et al., 2019; Lin & Beal, 2006; Long & Holtzman, 2019). Evidence suggests that build-up of toxic amyloid-beta (Aβ) in the brain is a pathogenic feature of AD. Other key characteristics include hyperphosphorylated tau, oxidative stress, and mitochondrial defects. Risk factors include the unmodifiable and the inevitable: genetics and aging, but some risk factors, such as diet, sleep, and trauma to the brain, are modifiable (Thelen & Brown-Borg, 2020).

AD is pathologically characterized by defects such as impaired mitophagy, reduced size of neurons, synapse starvation, and ATP depletion in the hippocampus (Fang et al., 2019). This brain area is important for memory function, though other cognitive functions such as communication and motor control are also impaired in AD due to defects in the cerebral cortex. The Alzheimer's Association estimates the current cost of care for people with AD to be nearly $600 billion annually, through both healthcare and family support channels (Alzheimer's Association, 2022). With the projected growth of incidence and prevalence of AD, there are ethical, scientific, and fiscal reasons to search for ways to reduce morbidity.

Early diagnosis of AD can lead to reduced costs and more effective treatment, ultimately leading to better patient outcomes (Alzheimer's Association, 2022). This is because disease progression can occur in midlife, before obvious symptoms present themselves (Kivipelto et al., 2001). Extracellular plaques of peptides called amyloid-beta (Aβ) form as the disease progresses, and the brain's neurons react by reducing synaptic connections and retreating as glial cells react to the foreign plaques (Reitz & Mayeux, 2014). Aβ is formed adjacent to a normal physiological process when amyloid precursor protein (APP) is first cleaved by β-secretase instead of α-

secretase (Figure 1; Bachurin et al., 2017; Müller et al., 2008). Normally, APP is successfully reduced to small fragments that may support neural health (Müller et al., 2008). However, the amyloidogenic pathway creates the neurotoxic form of Aβ at either 40 or 42 polypeptides long, denoted as $Aβ_{1-40}$ and $Aβ_{1-42}$ respectively. These peptides remain insoluble outside of the cell, and build up as plaques, especially at synapses and along blood vessels (Bachurin et al., 2017; Tawfik et al., 2021; Verma et al., 2015).

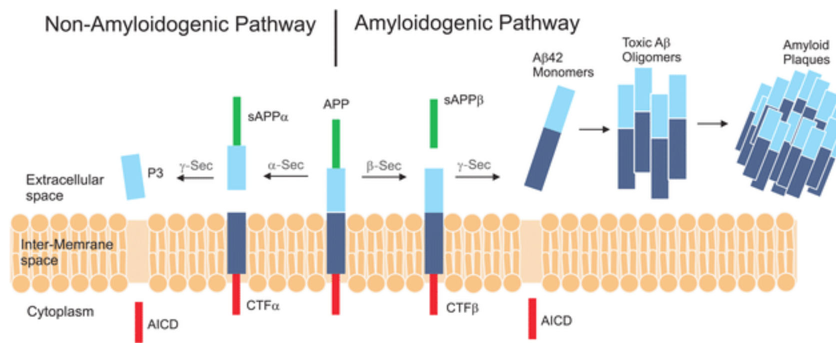


Figure 1: Amyloid precursor protein cleavage pathways.

Toxic amyloid-beta-forming and normal cleavage pathways of amyloid precursor protein (Figure from Bachurin et al., 2017).

Although Aβ is associated with AD, the density of Aβ plaques and the severity of AD is not strongly correlated (Chen et al., 2017). What research has suggested is that the smaller free oligomers of Aβ (AβO), which can insert into the plasma membrane and organelles such as mitochondria, endoplasmic reticulum, and lysosomes, can also have detrimental effects. Mitochondria defects are characteristic of AD, so this theory has some ground on which to stand (Cline et al., 2018; Pagani & Eckert, 2011). AβOs precede Aβ plaque formation, which could

explain why symptoms begin before significant plaque build-up (Fong et al., 2016; Iqbal & Grundke-Iqbal, 2010; Pagani & Eckert, 2011).

1.2 *Caenorhabditis elegans*

C. elegans is a choice model organism for research due to many factors. To anyone who has worked with the small nematode, it is of little surprise that *C. elegans* has been the research subject in three Nobel prizes so far (Greener, 2021). Some of the species' desirable features include a short generation time and lifespan, genetically tractable behaviors, a functionally equivalent muscular system to humans, many human orthologs, an easily modifiable environment and diet, and it poses no significant threat to the health of those who work with the nematode species (Brenner, 1974; Lam et al., 2021).

C. elegans has two sexes, male and hermaphrodite, which can be visually distinguished from one another. Both sexes produce sperm -- hermaphrodites produce approximately 300 sperm during meiosis before switching to oogenesis for the rest of the fertile period, males produce sperm that will outcompete that of hermaphrodites. Mating occurs using a male's tail, which is a phenotypically distinct structure to that of hermaphrodites. Mating between hermaphrodites cannot occur, but a hermaphrodite can self-fertilize herself in the absence of males. A protective eggshell forms around a fertilized egg and mitosis follows. These fertilized, protected eggs are stored in the mother's body for two or more hours before being laid. At twelve hours post-fertilization, the egg hatches the first larval stage of the worm, an L1. It continues to grow through larval stages L2, L3, and L4, before molting to the adult form. The late L4 stage is easily identifiable as the vulva presents as a white crescent with a central black speck. Another twelve hours after the final molt and the young hermaphroditic adult begins to lay its own eggs. (Riddle et al., 1997; Singson, 2001).

C. elegans can be raised at a variety of temperatures, with a noted effect on growth time. The egg-to-adult cycle takes only 3 days at 25°C, but lasts 5 days at 15°C (Brenner, 1974). An adult hermaphrodite will lay eggs for 3 days, though even once her supply of sperm is depleted she can survive for many weeks longer (Riddle et al., 1997; Singson, 2001). A single adult hermaphrodite can lays ~300 eggs, though introduced or natural mutations can limit or eliminate this number (Corsi et al., 2015; Singson et al., 1998). This large quantity of animals from one 'source' is useful for examining this population in assays with large sample size requirements. Because *C. elegans* is widely studied, mutations in many homologs of genes associated with human genetic diseases and biological processes can be found in readily available strains. Thus, *C. elegans* provides a simple model for aging and neurodegenerative diseases.

1.3 *C. elegans* models of Alzheimer's Disease

C. elegans has proven to be a useful system in studying age-related diseases such as AD, and can be used to model Aβ proteotoxicity (Fang et al., 2019; Fong et al., 2016; Teo, Lim, et al., 2020). These models use transgenes to express the $A\beta_{1\text{-}42}$ peptide, which is most abundant toxic Aβ species in the brains of AD patients, because *C. elegans* lacks the β-secretase, which cleaves APP (Dostal & Link, 2010; Drake et al., 2003). The Tanis lab commonly uses the GMC101 strain, which expresses $A\beta_{1\text{-}42}$ in the body wall muscles. The Aβ accumulates after shifting temperature to 25°C, and results in paralysis after a time, depending on other factors such as diet and genetics (Table 1). The model is robust because the worms exhibit a phenotype that is easy to score (Lam et al., 2021). Aβ strains have other pathological features of AD, such as mitochondrial morphology changes, reduced ATP levels, and increased reactive oxygen species (ROS) (Fong et al., 2016).

Since Aβ is found in the brain of AD patients and not the muscles, I employed another model to investigate the impacts of Aβ toxicity in relation to diet and aging. The GRU102 strain

expresses human $A\beta_{1-42}$ pan-neuronally and as such exhibits aging- and disease-related impairments. The GRU101 control strain acts like wild-type (Table 1) (Teo, Fong, et al., 2020). GRU102 provides a good model as defects at first are mild but increase as the animal ages and Aβ aggregates in the characteristic plaques of AD (Teo et al., 2019). One exhibited defect is impaired chemotaxis, the sensing and response to chemical signals, which forms a large part of my research. I first examined these strains regarding their healthspan and supplementation of nutrients. Healthspan refers to the period of life when there is an absence of morbidity and can provide a better model for disease progression than lifespan alone (Teo, Fong, et al., 2020). The conserved nature of metabolic pathways related to Aβ and the related pathologies demonstrate the benefit of using *C. elegans* to model Aβ toxicity (Lam et al., 2021).

1.4 Diet in Alzheimer's Disease and in *C. elegans*

While some risk factors for AD are non-modifiable, such as genetic predisposition and aging, some can be modified in ways that affect disease onset and severity. Some modifiable factors include head trauma, sleep quality, and diet. The Mediterranean diet has stood out as having potential protective effects against AD's symptoms, including decreasing mortality. This diet emphasizes consumption of fruits and vegetables alongside olive oil, fish, and other foods high in unsaturated fatty acids (Morris, 2009; Scarmeas et al., 2006; Yusufov et al., 2017). Other diets that are high in saturated fats and carbohydrates such as glucose may increase risk for developing AD (An et al., 2018; B. Lin et al., 2016; Taylor et al., 2019). Things like fats, carbohydrates, and protein are considered macronutrients. The human body requires these in relatively large quantities to sustain life to provide energy. On the other hand, micronutrients are ingested in much smaller quantities and do not directly provide energy. However, micronutrients include vitamins and minerals that are also essential to life (*What Are Macronutrients and Micronutrients?*, 2022).

Humans are complex creatures with complex diets, and on top of varied dietary intake, a diverse gut microbiome contributes to metabolism, nutrient composition, and can influence factors in health and disease (Cresci & Bawden, 2015). In laboratory settings, it is relatively easy to study the effects of diet in the *C. elegans* model because *C. elegans* eat a very controlled and simple diet of *Escherichia coli*. There is also no gut microbiota to influence nutritional uptake, as in humans (Lam et al., 2021). In short, researchers can precisely control dietary intake of *C. elegans* to examine the effect of specific nutrients.

1.5 SAM/Methionine Cycle

The methionine/S-adenosylmethionine (SAM) cycle is one part of the one-carbon (1C) metabolic network, others being the folate cycle and methionine remethylation and transsulfuration pathways. 1C metabolism is essential to the synthesis of DNA, amino acids, and phospholipids, and can be regulated through dietary supplementation of controlling substrates and cofactors in the pathway, notably vitamin B12 (or simply B12) and choline (Clare et al., 2019; Lyon et al., 2020). Vitamin B12 is an essential cofactor for the enzyme methionine synthase, which converts homocysteine to methionine (Figure 2). The amino acid methionine is essential and is metabolized to form S-adenosylmethionine (SAM), which is the most abundant methyl donor in cells and acts in DNA methylation and phosphatidylcholine (PtdCho) synthesis. PtdCho can be formed by SAM-dependent methylation of phosphoethanolamine or directly from choline through the Kennedy Pathway (Figure 2). PtdCho is the most abundant plasma membrane phospholipid and changes in the PtdCho levels impact gene transcription to regulate metabolism (van der Veen et al., 2017).

Human studies of AD have suggested a link to 1C metabolism and the disease, with evidence that low levels of vitamin B12 are a risk factor for AD (Mohajeri et al., 2015). B12 is a required cofactor for the enzyme methionine synthase, which generates methionine from

homocysteine, a molecule found to be in high levels in the plasma of AD patients (Clare et al., 2019; Mohajeri et al., 2015). Clinical trials have been completed to show that B12 supplementation can preserve cognition and slows brain atrophy (Kang et al., 2008; Smith et al., 2010). Since B12 deficiency increases with age (Green et al., 2017), and since evidence is unclear on how B12 supplementation prior to or during cognitive decline is affected by previous deficiencies (Clarke et al., 2014; Ford & Almeida, 2019), further research into the effects of vitamin B12 on cellular function is necessary.

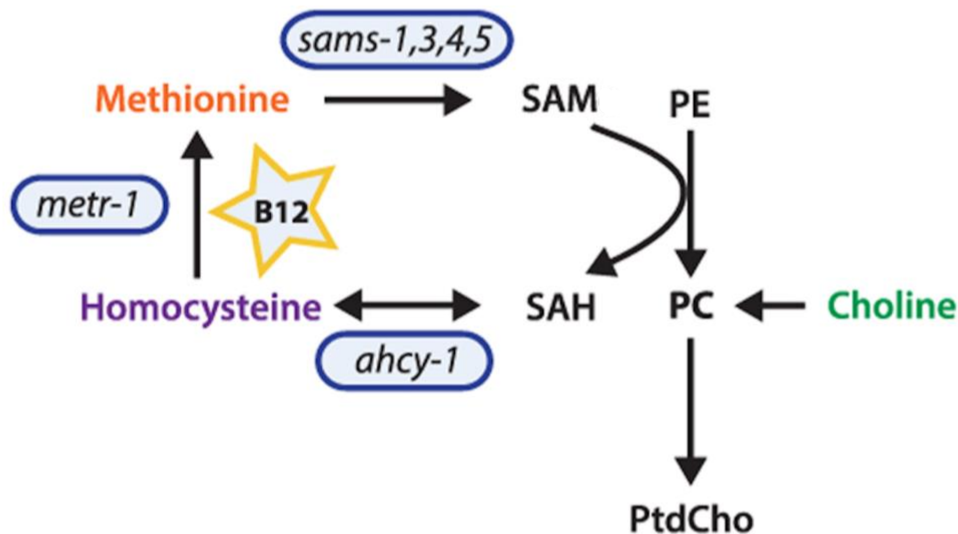


Figure 2: Phospholipid synthesis and the methionine/SAM cycle.

Protective effects of B12 are dependent on the methionine/SAM cycle. SAM, s-adenosylmethionine; SAH, s-adenosyl-l-homocysteine; PE, phosphoethanolamine; PC, phosphocholine; PtdCho, phosphatidylcholine. (from Lam et al., 2021).

Dr. Andy Lam in the Tanis lab experimented with B12 supplementation and found that in *C. elegans* that express Aβ in the body-wall muscles, there was a protective effect against paralysis (Lam et al., 2021). Additionally, since PtdCho is found to be reduced in AD patients

(Whiley et al., 2014), supplementation with the choline precursor molecule was examined by Dr. Lam. Again, there was a protective effect with choline supplementation (Lam et al., 2021). Choline was used instead of PtdCho directly as PtdCho-infused agar plates tended to be lethal to the animals grown on them. These results led to my research with *C. elegans* that express Aβ in neurons to investigate whether supplementing with these two nutrients is protective in a neuronal model.

1.6 Fatty Acid Synthesis

In a previous RNA sequencing analysis completed in the Tanis lab, the *fat-5* and *fat-7* genes were downregulated when Aβ animals were given a diet rich in vitamin B12. Transcription of these genes are upregulated when high levels of B12 in turn create high levels of PtdCho, which inhibits the transcription factor SBP-1 in *C. elegans*. SBP-1 increases transcription of genes including *fat-5* and *fat-7* (Walker et al., 2011). This suggests that B12 may impact fatty acid synthesis and lipid biology. Both genes produce stearoyl-CoA Δ9 desaturases, which play a part in converting saturated fatty acids into monounsaturated fatty acids. The difference in the two enzymes is that FAT-7 can act on 16- and 18-carbon chain fatty acids while FAT-5 only acts on the 16-carbon chain substrate. FAT-6 acts in the same way as FAT-7 (Figure 3; Watts, 2016). Loss of function mutations to these two genes of interest could give insight into how fatty acid content relates to Aβ-proteotoxicity and B12 supplementation.

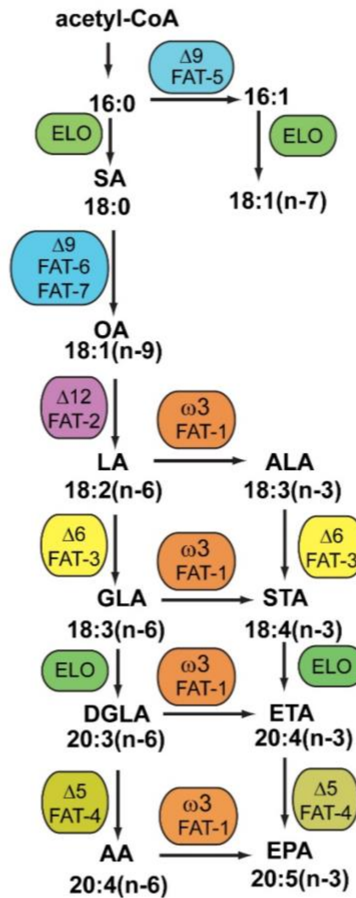


Figure 3: Fatty acid synthesis in *C. elegans*.

The role of FAT-5 and FAT-7, among other proteins, in lipid biosynthesis. (Figure from Watts, 2016)

1.7 Objectives and Hypotheses

Previous research in the Tanis lab has demonstrated how vitamin B12 and choline supplementation can reduce bioenergetic defects and slow the rate of paralysis normally seen in a strain of *C. elegans* expressing Aβ in the body-wall muscles (Lam et al., 2021). I hypothesized that this rescue effect would also be seen in *C. elegans* expressing Aβ pan-neuronally during a

chemotaxis assay, and that this model would more accurately represent human AD. My objectives to complete these assays included mastering the assay process and generating self-sterile strains of the Aβ and control strains. Once these initial assays were completed, I performed conditioning (learning and memory) assays with animals raised on a diet ±B12 supplementation. I hypothesized that B12 would help the Aβ animals 'remember' the aversive condition and therefore be beneficial.

Additionally, I sought to determine how altering fatty acid synthesis impacted the effect of vitamin B12 on the *C. elegans* strain expressing Aβ in the body-wall muscles. I used a traditional mating genetic cross to create Aβ animals that lacked *fat-7*, while I employed CRISPR-Cas9 technology to modify the genome and knockout *fat-5* in the Aβ animals. The null hypothesis for *fat-7* was supported when a paralysis assay with the newly created strain did not show any change in time-to-paralyze with B12 supplementation. One paralysis assay trial with *fat-5* similarly indicates that the dietary shift is not eliminated and that health with regard to B12 status is not affected by loss of the *fat-5* gene.

2. Methods

2.1 *C. elegans* Maintenance

The nematodes were maintained as a live supply on the agar-based nematode growth medium (NGM) contained in plates with lids. The media was autoclaved to sterilize prior to being poured into plates and standard additives were mixed in when the solution cooled to approximately 70°C (Brenner, 1974). A mechanized plate pouring pump dispensed standard amounts of the media into each plate. 6cm maintenance plates required 10mL of media, whereas 9cm assay plates required 25mL. Once solidified, an *E. coli* food source for the animals was dispensed on top of the agar. I used the OP50 strain grown in B Broth from a single colony source. 200µL was used for 6cm plates and 800µL was used for assay growth plates, with the latter requiring a gentle tilting of the plate to spread the bacteria across the middle of the agar. OP50 is unique in that it lacks uracil, so excessive growth of the bacteria was inhibited and worms on the plate remained visible and well fed (Brenner, 1974). As the animals used their food source their population required transferring to fresh plates. To do so, 3-5 worms were transferred using a platinum wire affixed to a glass handle. The wire was flamed prior to and after use to remove potential contamination. Day-to-day storage of the plates depended on the genetic background, at either 15°C or 20°C.

Table 1: *C. elegans* strains

Strain	Gene/Allele	Description	Maintenance Temperature
N2 (Bristol)	wild type	Strain cultivated from soil.	15°C or 20°C
GMC101	*dvIs100* [unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP] V	Expresses Aβ_{1-42} in body wall muscles.	15°C or 20°C
BX153	*fat-7 (wa36)* V	Knockout of one stearoyl-CoA 9-desaturase gene, involved in fatty acid synthesis.	15°C or 20°C
UDE212	*fat-7 (wa36)* V; *dvIs100* [unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP] V	Product of genetic cross between GMC101 and BX153; homozygous positive for *fat-7* deletion.	15°C or 20°C
GRU101	*gnaIs1(myo-2p::yfp)* IV	Control strain for pan-neuronal Aβ_{1-42} (GRU102). WT phenotype with pharyngeal YFP expression.	15°C or 20°C
GRU102	*gnaIs2(myo-2p::YFP+unc119p::Abeta1-42)* III	Expresses pan-neuronal Aβ_{1-42}. Impaired neuromuscular and sensorimotor behavior.	15°C or 20°C
BA671	*spe-9 (hc88)* I	Self-sterile hermaphrodites at 25°C.	15°C
BA963	*spe-27 (it132)* IV	Self-sterile hermaphrodites at 25°C.	15°C
UDE217	*spe-9 (hc88)* I; *gnaIs1(myo-2p::yfp)* IV	Control strain for pan-neuronal Aβ, self-sterile at 25°C.	15°C
UDE219	*spe-9 (hc88)* I; *gnaIs2(myo-2p::YFP+unc119p::Abeta1-42)* III	Expresses pan-neuronal Aβ, self-sterile at 25°C.	15°C
UDE272	*fat-5(tm420)* V; *dvIs100* [unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP] V	Product of CRISPR-Cas9 deletion of *fat-5* in GMC101 strain. *fat-5* is involved in stearoyl-CoA 9-desaturase activity.	15°C or 20°C

2.2 Supplementing NGM Plates

In addition to the standard additives of Nematode Growth Medium (NGM) agar plates, additional nutrients and chemicals can be mixed in prior to pouring. I used plates made with vitamin B12 and with choline to test the effects of these nutrients on behavior during assays. B12 from the methylcobalamin analog (Sigma-Aldrich) was used at a concentration of 150 nM. Choline in the form of choline chloride (Sigma-Aldrich), was used at a concentration of 10mM. Previous literature has described how 5-fluoro-2′-deoxyuridine (FUDR; Sigma-Aldrich) can prevent the formation of progeny in an assay population (Teo, Lim, et al., 2020). This is advisable because progeny can affect the scoring of the assay. After some experimentation with concentrations that would allow my strains to survive, but still to prevent any offspring, I reached the concentration of 50µM, though at all tested concentrations some number of progeny still formed. This led to the need for self-sterile strains of the amyloid-beta animals, created by genetic crosses.

2.3 Genetic Crossing and Genotyping of *C. elegans*

Genetic crossing between *C. elegans* strains was achieved by mating males from one strain with hermaphrodites from the other. Males were spawned by incubating L4s at 30℃ for 6 hours. Then Mendelian genetics guides the cross, with care needed to the location of genes of interest on the 5 pairs of autosomal chromosomes, as genes on the same chromosome can be affected by recombination. The resulting hermaphroditic cross progeny were “cloned” onto their own plates where they were able to self-fertilize. When a population was suspected to be the product of the cross – with both genes of interest expressed, one of which was often indicated by a fluorescent marker – the remaining gene(s) could be genotyped to confirm identity.

For my cross between the *fat-7* deletion strain and the strain harboring the *dvIs100* transgene, I used SuperSelective genotyping due to the nature of the point mutation in the *fat-7*

mutants which I needed to identify (Table 2). This polymerase chain reaction (PCR) method is described by (Touroutine & Tanis, 2020).

My crosses to introduce a self-sterility mutation to animals expressing Aβ pan-neuronally involved standard duplex PCR genotyping to quickly identify animals with the Aβ mutation (Table 2). To screen for the sterility mutation, animals were shifted from 15℃ to 25℃, where animals grown in the higher temperature would produce no progeny.

Table 2: Primers for Genotyping

Allele	WT	Mutant	Common	Description	Annealing Temp
fat-7 *(wa36)*	gtcgagtttgcctcctacga ggataacttG	gtcgagtttgcctcctacga ggataactt**A**	ggattcggtatcac agctgg	SuperSelective, common reverse	56.5℃
fat-5 *(tm420)*	ggctcaggcatatgctcag	gctctaggctgacgtactg	ctaaacagttcctg gcggc	Multiplex, common forward	60℃

2.4 Paralysis Assay

The UDE212 *fat-7* deletion strain was used by Andy Lam, a PhD student in the lab, in an assay described in (Lam et al., 2021) and in his 2022 dissertation (Table 1). This paralysis assay took advantage of the amyloid-beta expressed in the body wall muscles to paralyze the animals as an indicator of health. Stress factors, most notably the combination of Aβ-proteotoxicity and heat lead to such paralysis. Animals were synchronized to the same life stage and grown at 20℃ until late in the L4 stage, when they were shifted to 25℃. After 20 hours had elapsed, the number of paralyzed and non-paralyzed animals were quantified every 2 hours. The UDE212

strain was compared to GMC101 on both OP50 and HB101 bacteria, with the difference in strains being the *fat-7* deletion. Three biological replicates were performed per strain and condition. The UDE272 strain, which contains the *fat-5* mutation and Aβ transgene was compared to GMC101 in the presence or absence of vitamin B12, so the difference in these strains being the *fat-5* knockout (Table 1). Only one replicate was obtained for this comparison and counting stopped after all groups reached the median paralysis time. This time gives an indication of how robust a strain is in delaying paralysis, with longer times indicating that some condition limits the negative effects of Aβ in the body wall muscles. These data were used in Kaplan-Meier survival plots to determine median time to paralysis, using GraphPad Prism 9.

2.5 Chemotaxis and Conditioning Assays

The chemotaxis protocol has been adapted from (Margie et al., 2013) and the conditioning details from (Fang et al., 2019) to fit my own trial-and-error, available resources, and *C. elegans* strains.

PART 1: Preparing the Worms

1. Bleach preparation to synchronize worms to the same life stage.
    a. For each strain to be assayed, pick 7 L4 animals onto each of 4, 6cm plates.
    b. After ~4 days at 20°C or ~7 days at 15°C, plates will be saturated with adult animals containing eggs.
    c. Prepare bleach solution in fume hood by combining 37.5mL deionized H_2O, 10mL bleach, and 2.5mL 10N NaOH in a 50mL conical tube.
    d. Using a plastic transfer pipet and 3 rinses of 1x M9 buffer, wash animals into a 15mL conical tube.
    e. Centrifuge for 1 minute at 2000rpm.

f. Remove supernatant with a transfer pipet and add 10mL of bleach solution.
g. Repeatedly invert the tube for 4 minutes then centrifuge for 1 minute at 2000rpm.
h. Carefully pour off supernatant and eggs will stick to the inside of the tube.
i. Wash the pellet three times by adding 1x M9 buffer and centrifuging, then removing supernatant and repeating.
j. Using 1x M9, bring the total volume to 10mL and place on a rotator overnight at 15°C to let the eggs mature into L1 worms.

2. Plate 200 animals each onto 9cm growth plates with or without supplementation.
3. Store plates at 25°C.
4. At 3 days after plating, wash with 1x M9 buffer onto fresh plates.
5. At 6 days after plating, the 5-day-old adults are ready to be assayed.

PART 2: Preparing the Assay Test Plates (Figure 4)

1. Use a marker to draw lines dividing the bottom of a 9cm plate into 4 equal quadrants.
2. In the center of the plate draw a circle with a diameter of 8mm. This inner circle delineates an area where movement is not scored, which prevents immobile worms from skewing the data.
3. Alternating in each quadrant, mark either "T" for "Test" or "C" for "Control" along with a point 3.5cm from the origin to indicate where treatment and other additives should be placed. The alternating quadrants should remove any bias from imperfect placement of animals at the origin.

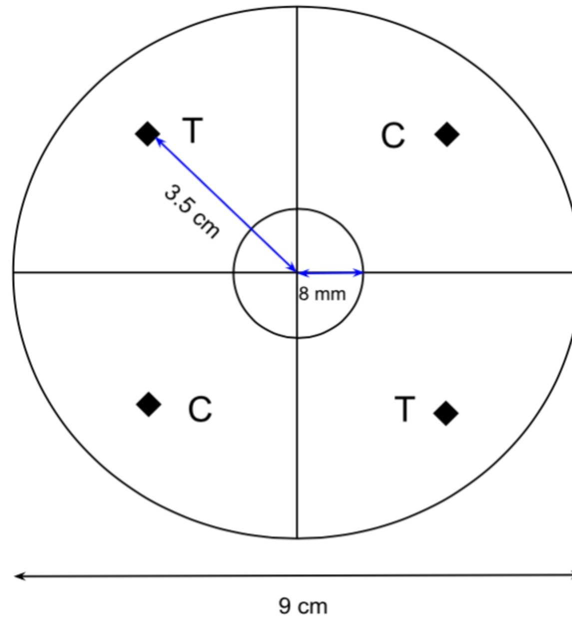


Figure 4: Assay Test Plate Layout.

The assay test plate is divided into quadrants alternating treatment and control. A starting circle omits any animals that did not move from the final count.

(PART 2.5): Conditioning

The conditioning steps have been adapted from (Fang et al., 2019). These extra steps were not completed during initial chemotaxis trials with choline and vitamin B12.

1. Using a plastic transfer pipet, wash the conditioning group animals off their growth plate with 3 rinses of S Basal into a 15mL conical tube.
2. Centrifuge for 1 minute at 2000rpm.
3. Remove the supernatant with a transfer pipet and wash twice more by adding S Basal and finally once with water. Do not skip any washes because residual bacteria may interfere with the assay.
4. Remove supernatant so that the volume of the worms is ½ of the volume in the tube.
5. Add 5μL pure isoamyl alcohol to the lids of unseeded 9cm NGM plates.

6. Plate animals from tubes to the conditioning plates, condition for 90 minutes.
7. Using water, wash the worms off the conditioning plates into new conical tubes before assaying, starting at step 4 in part 3 of this protocol.

PART 3: Running the Assay

1. Using a plastic transfer pipet, wash animals off their growth plate with 3 rinses of S Basal into a 15mL conical tube.
2. Centrifuge for 1 minute at 2000rpm.
3. Remove the supernatant with a transfer pipet and wash twice more by adding S Basal and finally once with water. Do not skip any washes because residual bacteria may interfere with the assay.
4. Remove supernatant so that the volume of the worms is ¾ of the volume in the tube.
5. Pipet 1µL of 1M sodium azide on the agar at each labeled point. Sodium azide acts as an anesthetic and arrests the worms upon reaching a quadrant.
6. Wait 1-2 minutes for the sodium azide to set.
7. Pipet 1µL of isoamyl alcohol (1:100 dilution in water) on the agar at each labeled "T." Pipet 1µL of water on the agar at each labeled "C."
8. Wait another 1-2 minutes for the treatments to set.
9. Using a glass pipette, pipet the worm pellet of 100-200 worms onto the origin of the assay plate.
10. Tilt plate lids ajar in fume hood to dry excess liquid for 5 min. If worms clump together in the origin after 5 min, use platinum pick to spread the worms out within the origin circle.
11. After 60 min (90 min if conditioning assay), count the worms anesthetized in each of the labeled quadrants.

PART 4: Determining the Chemotaxis Index

1. Calculate the chemotaxis index (CI) for each assay plate using the equation in Figure 5. This will yield a chemotaxis index between -1.0 and +1.0. A +1.0 score indicates maximal attraction towards the test reagent and represents 100% of the worms arriving in the quadrants containing the test reagent. A -1.0 score indicates maximal repulsion.

$$Chemotaxis\ Index\ (CI) = \frac{\#\ Worms\ in\ Both\ Test\ Quadrants - \#\ Worms\ in\ Both\ Control\ Quadrants}{Total\ \#\ of\ Scored\ Worms}$$

Figure 5: Chemotaxis Index Equation.

This equation quantifies attraction or repulsion from a treatment from a score of +1 to -1 respectively, and a value of 0 indicating no preference between treatment and the control.

PART 5: Data Analysis

1. Use GraphPad Prism 9 or similar data analysis software to compile data from biological replicates and to make graphs.
2. Use t-test and one-way ANOVA test to test statistical significance.

3. Results

3.1 Genetic Crosses

The *fat-7* point mutant cross with the *dvIs100* Aβ transgene was difficult and time-consuming because both *fat-7* and *dvIs100* are on chromosome V in the *C. elegans* genome, and therefore recombination likely was the reason why double mutants were hard to create (Figure 6). After designing the SuperSelective primers for the *fat-7* cross, I first ran a gradient PCR to find the optimal annealing temperature that would enable me to distinguish mutant from wild-type animals. 56.5°C was found to be best for wild-type and mutant primers. Then, I set up a cross with *dvIs100* males and *fat-7* hermaphrodites. Cross progeny in the F1 generation were identified as animals with GFP fluorescence in the intestine, since the *dvIs100* transgene causes expression of not only Aβ in the body-wall muscles, but also intestinal GFP, which is a visible marker that is used to follow the transgene. Animals that did not have GFP fluorescence in the F2 generation were screened out because they lacked the transgene. I then isolated DNA from population lysis of F3 animals and performed PCR to identify animals that had had the *fat-7* mutation. Twice I had to restart the cross and overall I had to clone out over 450 animals from hermaphrodites that had GFP and potentially showed mutant bands in the agarose gel imaging. Eventually, I identified and confirmed a plate that contained animals that were homozygous for both the *fat-7* point mutation and *dvIs100* Aβ transgene (Figure 7).

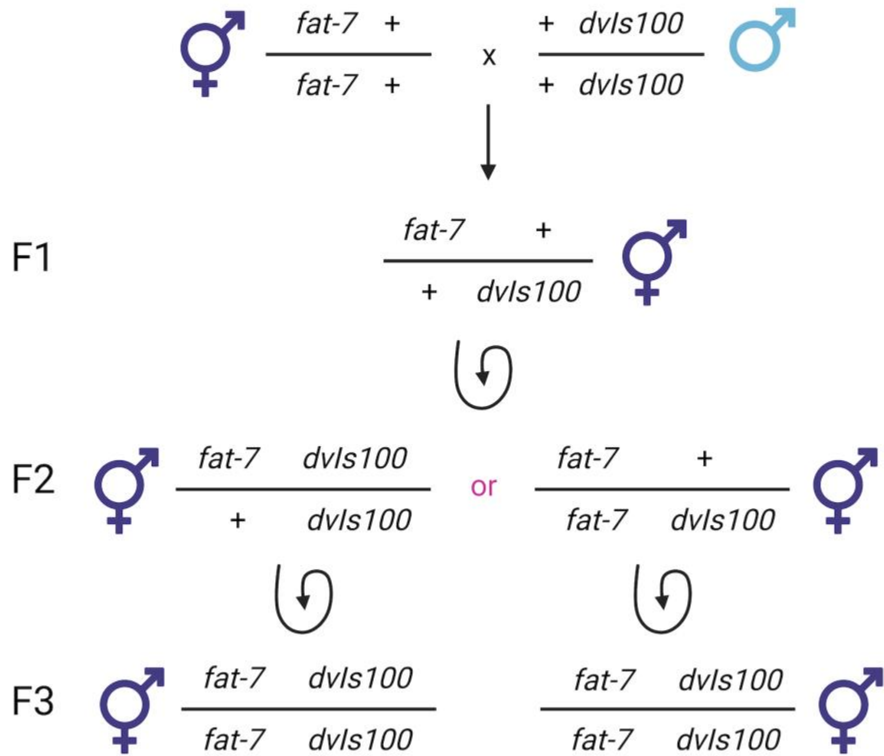


Figure 6: Genetic cross schematic between *fat-7* point mutant and *dvIs100* transgene.

This diagram shows the cross plan, illustrating how both genes are on the same chromosome and that three generations were needed to reach the double homozygote.

Figure created with BioRender.com

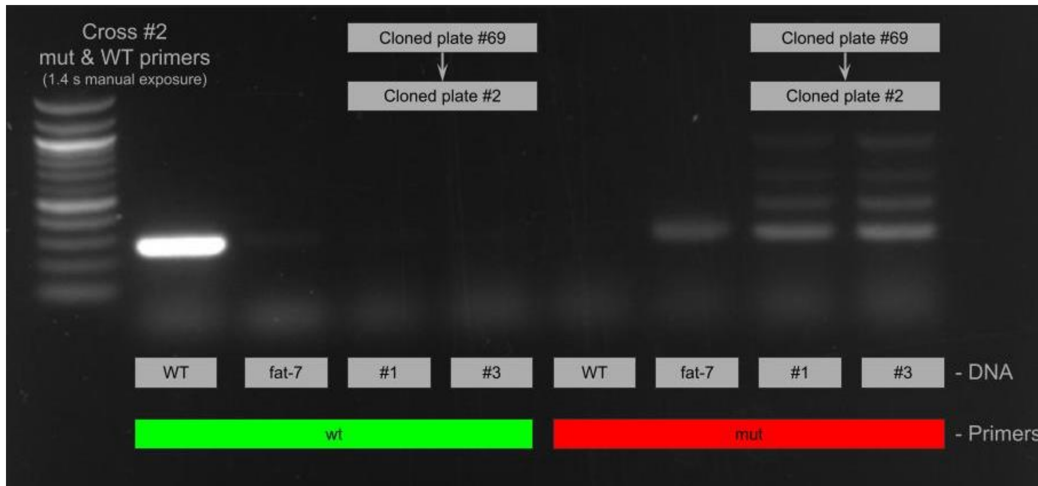


Figure 7: Gel image from successful cross of *fat-7* and *dvIs100*.

The gel image that showed the final product of the cross (wells #1 and #3), which then needed to be confirmed; it was not a heterozygote for *fat-7*.

3.2 The *fat-7* mutation does not impact Aβ-induced paralysis

The loss of *fat-7* in the UDE212 strain leads to changes in fatty acid content due to the absence of an essential Δ-9 fatty acid desaturase used in monounsaturated fatty acid synthesis (Deline et al., 2013; Watts & Ristow, 2017). The paralysis assay was used to determine if fatty acid content influenced Aβ-proteotoxicity. Diet was controlled using two different *E. coli* food sources, OP50 and HB101, which have low and high vitamin B12 content, respectively (Lam et al., 2021). Although there was a significant difference in median paralysis between OP50 and HB101 diets in both GMC101 and UDE212 animals, there was no significant difference between GMC101 and UDE212 on either bacterial diet (Figure 8). This indicates that loss of *fat-7* does not modify Aβ-proteotoxicity positively or negatively. Further, since the dietary shift between bacterial strains still exists this suggests that changes in *fat-7* expression do not underlie the protective impact of dietary vitamin B12. One explanation for this is that *fat-7* might affect the

wrong branch in fatty acid synthesis. These data lead to my work with *fat-5* as an alternative gene of interest.

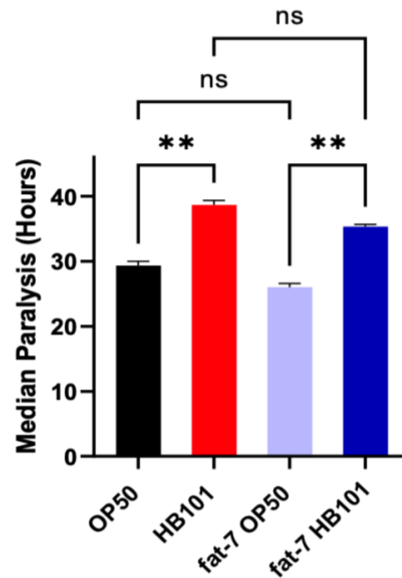


Figure 8: *fat-7* deletion had no effect on median paralysis.

Vitamin B12 was protective in both wild type and *fat-7* mutant animals; **p<0.01
(Figure from Dr. Andy Lam)

3.3 The *fat-5* mutation does not impact Aβ-induced paralysis

Expression of the FAT-5 fatty acid desaturase is also impacted by dietary vitamin B12. The clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system of genome editing was employed to introduce a *fat-5* knockout mutation into the GMC101 strain which contains the *dvIs100* Aβ transgene to create our UDE272 strain (Table 1). I used this strategy since the *fat-5* mutation and *dvIs100* Aβ transgene are on the same chromosome and performing a cross by traditional methods likely would have been difficult – like the *fat-7* cross proved to be. CRISPR was performed according to standard practices with *C. elegans* as described in

(Dickinson & Goldstein, 2016; Kim & Colaiácovo, 2016). First, Dr. Tanis injected the CRISPR RNA, tracerRNA, and Cas9 protein into the syncytial gonad of a hermaphrodite, the location where oocytes are budded off from a precursor cell with multiple nuclei (Samuel et al., 2001). The CRISPR RNA recognizes the sequence of interest and trans-activating crispr RNA (tracrRNA) guides the endonuclease Cas9 to make the necessary snips. A co-CRISPR RNA was also used to produce a visible phenotype to enable visually screening for animals that had a genome edit. Our co-gene was *dpy-10*; homozygote mutants are "dumpy" because they are shorter and rounder than wild-type, and heterozygote are "rollers," because they move in tight circles on their agar plates. Progeny from the injection were allowed to have their own offspring and then I picked rollers for multiplex genotyping. Then I cloned non-dumpy, non-roller progeny from any plates of F1 heterozygotes of *fat-5*, allowed them to have their own offspring, then genotyped. This process was repeated until wild-type-looking animals that were homozygous for the *fat-5* deletion and that do not produce dumpy or roller progeny (the co-CRISPR phenotype) were obtained. Even though the two genes of interest were on the same chromosome, the creation of the new UDE272 strain was relatively straightforward and only required three rounds of genotyping and phenotypic analysis, including a confirmatory round (Figure 9).

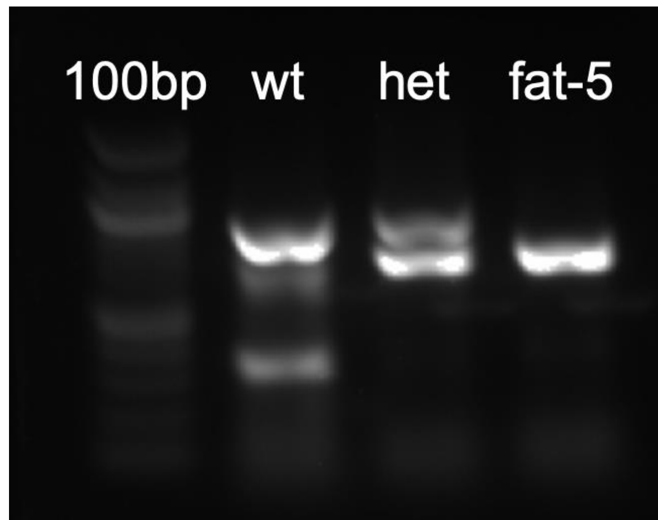


Figure 9: Isolation of a new *fat-5* deletion mutant.

Confirmatory gel image showing the UDE272 strain that is homozygous for the *fat-5* deletion; these were non-dumpy, non-roller, and thus no longer had the co-CRISPR edit.

The UDE272 strain expresses Aβ in the body wall muscles and has a *fat-5* knockout. Since I recently generated this strain, I was only able to complete one paralysis assay, but it indicates that the vitamin B12-dependent shift in paralysis is not eliminated in the absence of *fat-5*. Instead of varied bacterial diets, I used the condition of B12 supplementation to determine if this sole nutrient would produce a shift. The median paralysis time for Aβ animals supplemented with B12 tracks with previous experiments, but the unsupplemented group of these animals had a more delayed time than previously observed (Lam et al., 2021). This could be due to many factors, including a low sample size or inconsistency with the age of the plates the animals were grown on. However, the *fat-5* animals demonstrated times that are expected for normal Aβ animals in the presence and absence of B12 (Figure 10).

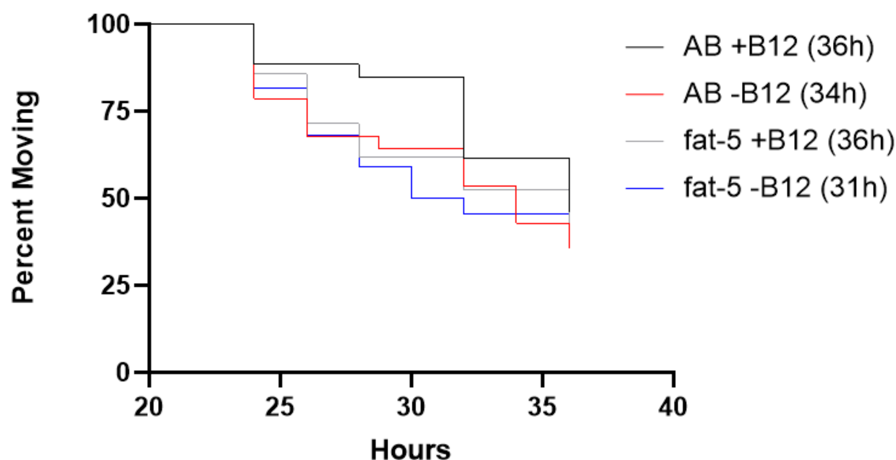


Figure 10: Aβ-induced paralysis in wild-type and *fat-5* mutant animals. Median time to paralysis in parentheses.

Early experimentation with *fat-5* KO in Aβ animals indicates that the dietary shift is not eliminated, though the value for the unsupplemented control was different from previous results.

3.4 Chemotaxis Assay with Choline and Vitamin B12 Supplementation

The Tanis lab has extensively examined Aβ-proteotoxicity in body-wall muscles of *C. elegans* and has found vitamin B12 and choline to be beneficial nutrients in reducing Aβ-related detriments. I wanted to test these nutrients in a neuronal model, which I believed would more accurately reflect human AD due to the location of Aβ build-up. The chemotaxis assays first began by researching the technique and previous use of animals expressing pan-neuronal Aβ in chemotaxis assays by the Gruber and Chin-Sang labs (Margie et al., 2013; Teo et al., 2019; Teo, Fong, et al., 2020; Teo, Lim, et al., 2020). Since FUDR was used by the Gruber lab with the Aβ animals, I initially experimented with that chemical to prevent progeny formation. This is important because the assay was meant to compare effects between one cohort of animals as it

aged, so younger animals would essentially 'contaminate' a plate if they were present. The FUDR never fully prevented progeny formation, but these initial assays gave me more insight into the assay process. Most importantly, I was able to refine techniques for growing and plating the animals to achieve consistent and sufficient sample sizes. Additionally, I ran assays with the adult cohort at Days 1, 5, 8, and 10, and finally narrowed down the optimal time for assaying as Day 5 post fourth larval stage. This time was chosen as it seemed to demonstrate the biggest difference in behaviors between the mutant and control animals. Adults were also old enough to have aged, but Day 5 was before the natural negative effects of aging on locomotion were evident in both strains. Still, these data were still plagued with issues due to low and inconsistent sample size of animals and the fact that progeny could infiltrate a specific adult cohort.

Since the FUDR was not completely preventing progeny growth, I decided to use a different strategy and make the neuronal Aβ and control strains self-sterile. I crossed in a mutation that would allow for propagation of the animals at the permissive temperature of 15ºC, but that resulted in sterility when grown at the restrictive temperature of 25ºC. Dr. Amber Krauchunas of the University of Delaware's Biological Sciences Department suggested that a mutation in *spe-9* or *spe-27* would be suitable for this goal (Table 1). Through genetic crosses, I discovered that the *spe-27* mutation and the neuronal Aβ transgene are located on the same chromosome, so cross attempts with that sterility mutation were futile. Instead, *spe-9* was crossed into the neuronal Aβ and control strains (Figure 11) with great help from Rachel Wang, another undergraduate and Juntian Wei, a PhD rotation student in the Tanis lab. The self-sterile neuronal Aβ strain (UDE219) and control strain (UDE217) were used for all subsequent experiments (Table 1).

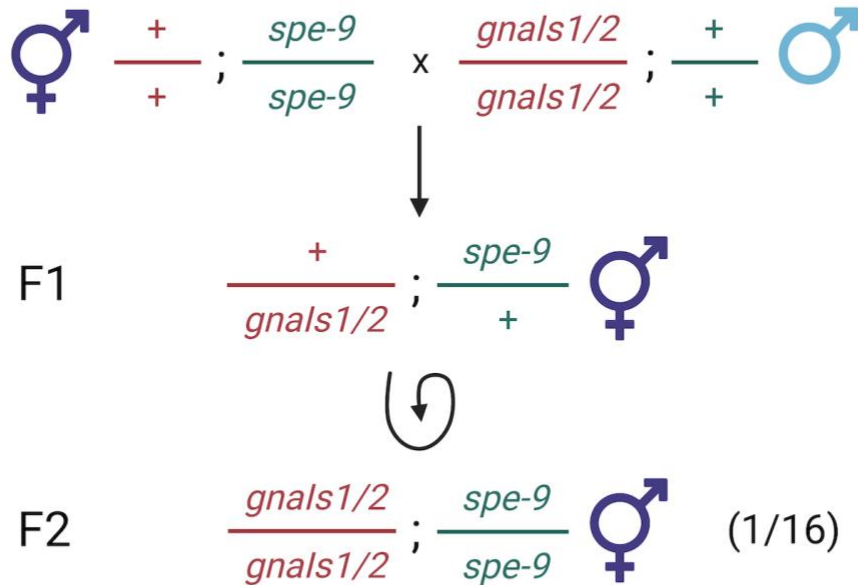


Figure 11: Genetic cross schematic between *spe-9* mutant and *gnaIs1* or *gnaIs2* transgene. (1/16) indicates Mendelian ratio of F2 progeny.

This diagram shows the cross plan, illustrating how the two gene loci are on separate chromosomes, and that only two generations were needed to reach the double homozygote. Figure created with BioRender.com

Once the sterile pan-neuronal Aβ animals were created, the trials with B12 and choline supplementation could begin. I divided this work with fellow undergraduate Rachel Wang; she took the B12 trials, and I used choline in my assays. Since it takes approximately two weeks of physical set-up for the assay and since the assay can take up to 3 hours, it was difficult to plan and execute the assays, but eventually we had five bio-replicates for each supplementation condition. What we found was that B12 supplementation rescued the chemotaxis defects observed in the pan-neuronal-Aβ animals, whereas choline supplementation had no significant effect on chemotaxis behavior (Figure 12).

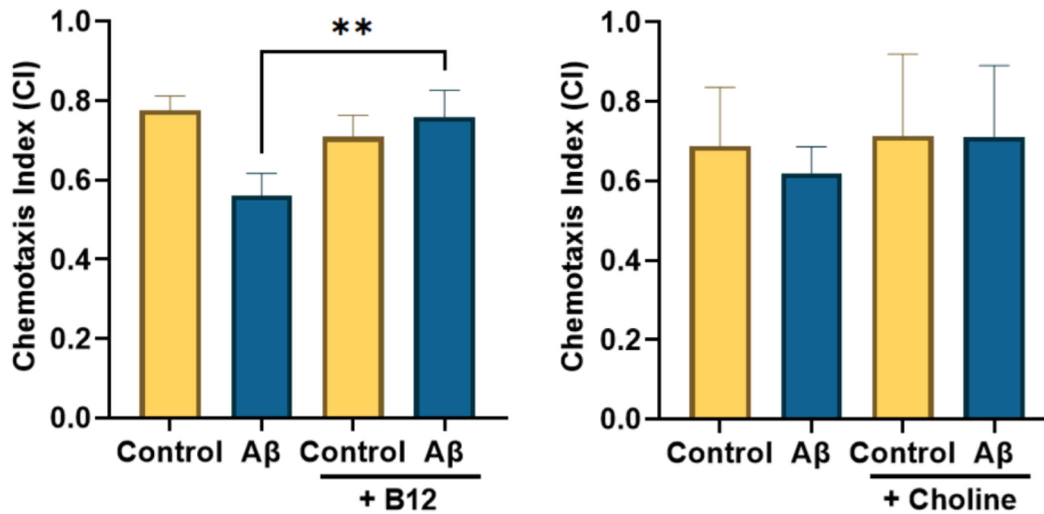


Figure 12: Chemotaxis assays with B12 and choline supplementation.

B12 supplementation rescued pan-neuronal-Aβ animals' defects, choline supplementation had no effect on chemotaxis behavior (B12 data from Rachel Wang). **$p<0.01$

3.5 Conditioning Assay

After the chemotaxis assay results indicated that B12 supplementation was protective in animals expressing Aβ pan-neuronally and that choline was not, it was logical to continue examining the effect of only vitamin B12. In keeping with the same basic assay structure, I hoped to modify the assay in ways described by previous literature to further examine memory and learning in *C. elegans*. This was achieved by aversive conditioning of the animals as described by (Fang et al., 2019), and with the protocol slightly modified in attempt to make it track with my previous chemotaxis assay methods. Five days after age-synchronized animals reach the fourth larval stage, they are either pre-exposed to IA or kept naïve, and then the groups are plated onto assay plates where they are scored for attraction or repulsion using a chemotaxis index. As in those assays, four trials were conducted of the conditioning assay with pan-neuronal-Aβ animals and with B12 supplementation. Overall, the data was not significant

between any group, possibly due to inconsistency between trials. Notably, B12 did not rescue Aβ animals in the naïve condition as was previously seen in the chemotaxis assays. Conditioning did lower the CI as was expected, but B12 "rescued" this change even though theoretically the healthier animals would have a lower CI. A graph depicting the individual data points as well as average values demonstrates how often one point was farther from the average than the others (Figure 13). This highlights the inconsistency of this assay as the outlying points are from various trials and because the average values are still off from my predictions based on previous data.

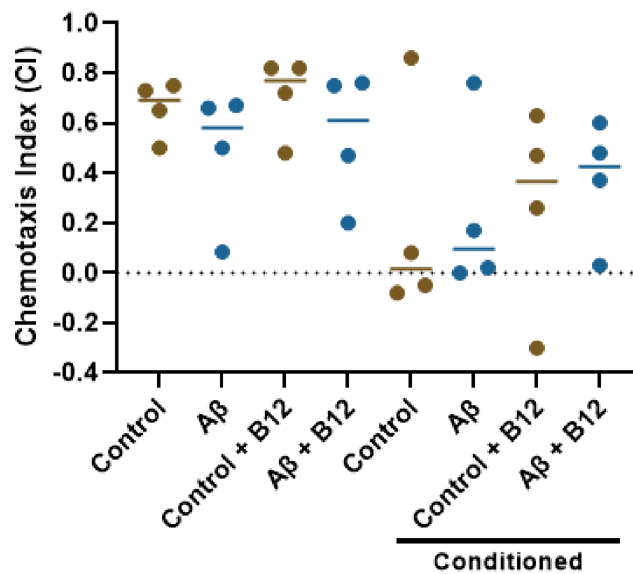


Figure 13: Chemotaxis index for animals exposed to aversive conditioning.

The average CI graph from the conditioning assays with individual data points shows how often just one point is off per condition, though the outliers are from various trials.

Examining the trials separately gives further insight into trends and takeaways from these data. In the first trial, the trend for non-conditioned (naïve) animals that was seen in the previous chemotaxis assays is absent. Instead, both Aβ and control animals had about the same CI when

unsupplemented and with B12 added. In this trial the conditioned groups similarly had one value off from the expected CI, this being the B12-supplemented control group, which was higher than predicted. This indicates that B12 was not helping the animals ‘remember’ the negative condition of starvation that was associated with the attractant isoamyl alcohol (Figure 14A). Trial two had expected values in the naïve groups, though according to the assay protocol the Aβ groups performed better, with lower CIs, than the control groups. This indicates that the assay may not be performing correctly or simply is not a good indicator for learning and memory in these pan-neuronal-Aβ animals (Figure 14B). The third trial demonstrated some expected trends, though the values in the naïve groups are off from previous data overall, and B12 did not rescue Aβ animals fully to the level of the control. Because of these changes to what previous data have shown as standard for the naïve animals in this type of assay, it can be expected that the conditioned values might similarly be inconsistent. However, these conditioning groups did show the expected trend, because B12 supplementation did lower the CI, and both conditioned control animal groups had lower CIs than the Aβ groups in this condition (Figure 14C). The fourth and final trial had some of its own oddities. Vitamin B12 did not rescue Aβ animals in the naïve condition as Tanis lab data has previously suggested is the case, and increased CI in conditioned Aβ animals, which goes against the theory behind this assay. Similarly, B12 increased CI in conditioned control animals, though the naïve control animals were not far off from previously attained CI values (Figure 14D).

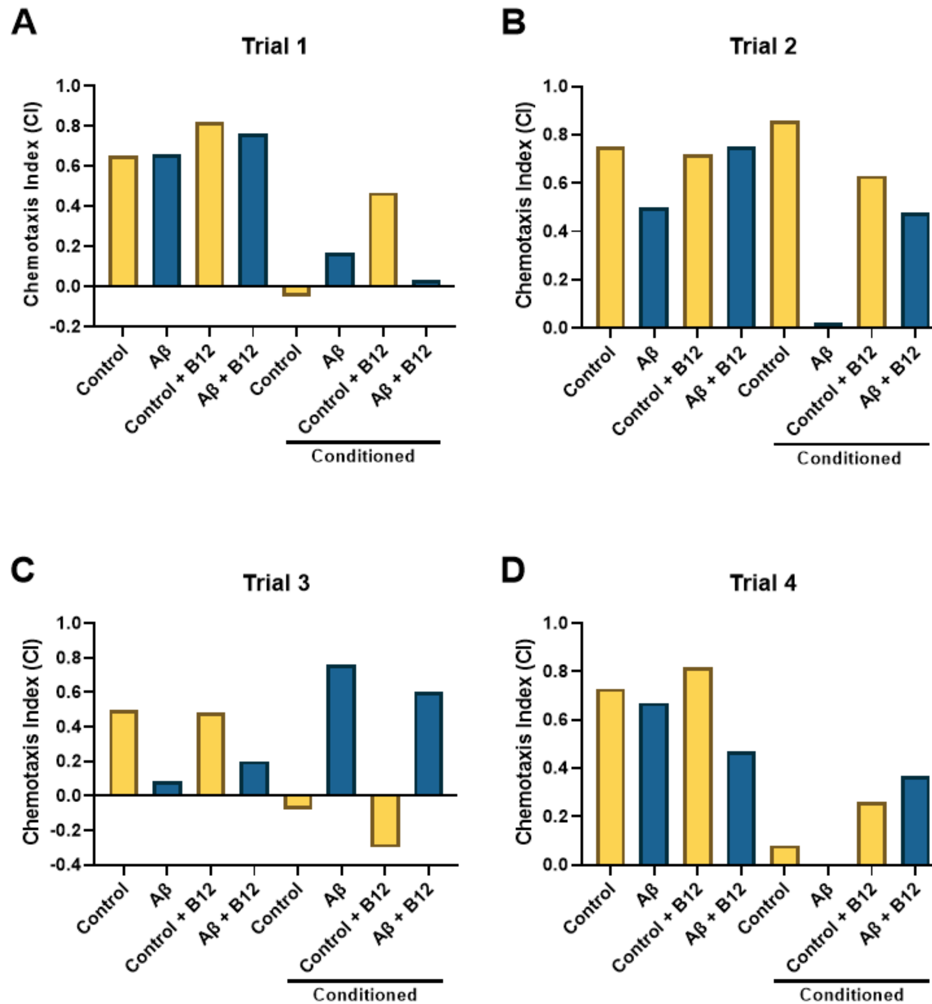


Figure 14: Individual trial results of conditioning data.

Individual replicate data from conditioning assay. **(A)** Conditioned results for the B12-supplemented control group were higher than expected in this first trial, and the naïve control group had a lower value than expected, at the same CI as the equivalent mutant group. **(B)** In trial 2, the naïve data was as expected, but the conditioning data did not match previous results or expectations. **(C)** Trial 3 had expected trends, but the values were shifted from previous successful experiments. **(D)** In the fourth trial, B12 appeared to not rescue the Aβ animals, and unsupplemented Aβ animals performed better than the controls.

The data for the control groups only does seem to suggest that conditioning does lower the CI, though this was not shown to be significant through my four trials due to variability in the data. Both supplemented and unsupplemented naïve groups had similar CI values, as did the conditioned groups with and without B12 (Figure 15).

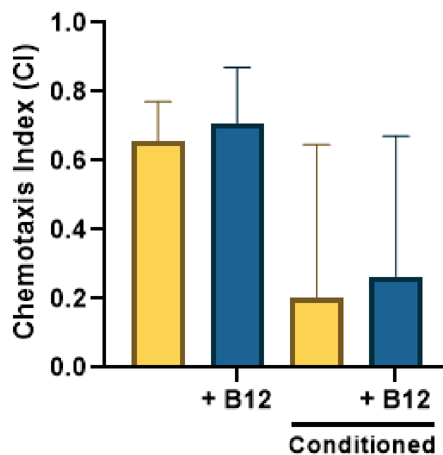


Figure 15: Control strain conditioning data.

Conditioning results with only the UDE217 control strain. Conditioning seemed to lower CI from naïve, though B12 did not lower CI in conditioned animals.

4. Discussion

The Tanis lab has previously demonstrated how vitamin B12 and choline supplementation can reduce bioenergetic defects and slow the rate of paralysis normally seen in a strain of *C. elegans* expressing Aβ in the body wall muscles (Lam et al., 2021). I investigated the protective impact of vitamin B12 in relation to stearoyl-CoA Δ9 desaturases FAT-5 and FAT-7, which act in converting saturated fatty acids into monounsaturated fatty acids (Watts, 2016). I created strains with loss of function mutations in these two genes and Aβ in the body wall muscles, so that they could undergo paralysis assays and be used to investigate how fatty acid content relates to Aβ-proteotoxicity and B12 supplementation. My second goal was to determine the effect of vitamin B12 and choline on animals expressing Aβ pan-neuronally, believing that this expression is more relevant to human AD.

I was involved in four genetic crosses of *C. elegans* to make novel strains with new combinations of gene knockouts and transgenes. The first strain created with UDE212, created by a traditional genetic cross between the GMC101 strain, which expresses the body wall muscle Aβ-expressing transgene *dvIs100*, and the strain BX153, which contains a knockout of the *fat-7* stearoyl-CoA 9-desaturase gene. GMC101 is the strain used in the Tanis lab for paralysis assays in the determination of diet on the health of the animals, and its transgene *dvIs100* is located on chromosome V. This presented a problem, since *fat-7* is also located on chromosome V, which likely led to the difficulties that occurred during the cross because of the increased likelihood that these two loci are inherited as a single unit. Traditional Mendelian genetic ratios were not present in the process of the cross due to this but cloning out large numbers of heterozygotes and potential heterozygotes eventually led to generation of the strain by genetic recombination. In hindsight, it would have been easier to generate this strain through CRISPR-Cas9 knockout of *fat-7* in the GMC101 strain, as was done for *fat-5*, however, CRISPR-Cas9 technology was not being used in the lab when I started this project.

UDE272 was created by performing CRISPR-Cas9 to generate a *fat-5* knockout, another stearoyl-CoA 9-desaturase gene in the parent strain GMC101. Because I used CRISPR-Cas9, the *fat-5* gene was immediately knocked out, along with a co-CRISPR *dpy-10* mutation to produce a visible phenotype, and I did not have to perform a genetic cross. Then this secondary co-CRISPR *dpy-10* mutation simply needed to be crossed out in favor of *fat-5;dvIs100* mutants.

The two different stearoyl-CoA 9-desaturase mutant strains (UDE212 & UDE272) were used in paralysis assays to determine the effects of fatty acid metabolism on Aβ-proteotoxicity. A graduate student, Andy Lam, completed trials with animals grown with two different bacterial food sources, and found that UDE212, which contains the *fat-7* mutation, did not eliminate the shift in time-to-paralysis that is present in GMC101 raised on HB101 versus OP50 *E. coli* strains. This supports the null hypothesis that *fat-7* deletion had no effect on median paralysis, possibly because it works only on one of two branches in acetyl-CoA metabolism. Although FAT-7 and FAT-6 act in the same way, previous research in the Tanis lab found that the shift remained after oleic acid supplementation, which would bypass both proteins, so *fat-6* does not pick up the slack of knocked-out *fat-7* (Lam et al., 2021). A gene that works on the other branch, *fat-5*, knocked out in UDE272, was then identified as another possible actor in eliminating the dietary shift seen in paralysis assays with *C. elegans* expressing Aβ in the body wall muscles. I completed one paralysis assay trial with this gene of interest, the data from which initially suggest that the dietary shift is similarly not eliminated with and without the condition of B12 supplementation. This could imply that this other branch of fatty acid synthesis might also not impact Aβ-proteotoxicity. Although I used a different growth condition for this paralysis assay, B12 supplementation is analogous to the difference in HB101 and OP50 diet because one of the ways in which these bacterial strains differ is in their B12 content. Of course, more trials with B12 and possible trials with these bacteria would be necessary to determine significance of these data. In the future, other genes involved in fatty acid synthesis and implicated to be upregulated in Aβ strains by RNASeq analysis should be examined in a similar way.

The paralysis assay is tricky to complete, not because it is labor intense, but because collecting a full set of data points can take over 20 hours of active participation in the experiment, and because the L4 animals must be picked at a precise time (t=0) the day before the assay. Since data is gathered every two hours, there is break time, but the more conditions involved in a trial, the more time is spent counting animals. Therefore, this assay needs careful planning for set up to ensure that the schedule works and that it is at an optimal time for the researcher to be in lab and to get enough rest before and after the assay.

Two crosses to make sterile strains for control and pan-neuronal-Aβ transgenes for chemotaxis assays were completed with assistance from Rachel Wang and Juntian Wei, and these strains (UDE217 & UDE219) allowed for easier assaying because no progeny were present to be confused with the tested cohort of animals when grown at the restrictive temperature. These crosses had some trial and error, but we specifically picked the *spe-9* gene to introduce sterility because it is on a different chromosome from both the mutant and control pan-neuronal-Aβ transgenes. For the most part, the difficulty lay in growing the animals to maturity to mate them, as the *spe-9* gene only produces progeny at lower temperatures, where *C. elegans* grows slower. Still, these described crosses ended up successful in producing strains to be used in experiments on diet and Aβ-proteotoxicity and memory and learning.

The chemotaxis and conditioning assays are even more difficult to plan and complete. Because the animals must at first be grown at the non-restriction lower temperature to allow progeny, set up takes exactly two weeks. The actual assay takes 3-6 hours, with the conditioning assay being longer and more intensive than the simple chemotaxis assay. In addition to this, the assay is resource intensive, so a solid inventory of both seeded and unseeded NGM plates as well as seeded B12 plates needed to be carefully maintained without letting the plates get old and desiccated. This same principle led me to keep making fresh batches of the buffer S Basal in attempt to maintain reliability between trials. These facts made planning and set-up high stakes, as often contamination of the growth plates or mistakes such as during bleach preparation could delay these assays by weeks.

When I first started the chemotaxis assays the protocol was different, and I had to experiment with different methods of washing, plating, and encouraging animals on the test plates to leave the starting circle. Originally the animals were not selectively genetically sterile, and instead we used FUDR in the growth plate medium to cause sterility as was described by previous literature (Teo et al., 2019; Teo, Lim, et al., 2020). I found that this was not effective enough to completely eliminate progeny, and the added step of creating these special plates with a dangerous chemical was undesirable. After many months, a working and replicable protocol was created and then adapted to the conditioning assay.

The chemotaxis assay trials were divided between another undergraduate in the laboratory, Rachel Wang, and me. All were planned and conducted together, but our division of labor between the two nutrients we examined made this more manageable. Rachel handled B12 while I used choline, which could lead to some reliability issues after I switched to B12 for the conditioning assays. Still, the same protocols were used for scoring the assay and preparing agar plates. I switched to B12 because the data suggested that choline did not have a significant effect on chemotaxis behavior, while B12 did significantly rescue Aβ animals to have a similar CI to that of the control. One reason choline might not rescue Aβ animals is because it is naturally produced by *C. elegans*, while B12 is not, but is an essential cofactor in the methionine/SAM cycle (Mullen et al., 2007; Rand, 2007; Zečić et al., 2019). Although choline did rescue nutrient deficient animals in previous paralysis assays (Lam et al., 2021), it is possible that supplementation of this nutrient does not affect pan-neuronally expressed Aβ-proteotoxicity as it did in GMC101 and related strains.

In all cases, there was no significant difference between CI values of unsupplemented control, supplemented control, and supplemented Aβ animals within one nutrient condition. This suggests that neither nutrient provides additional benefits to non-Aβ animals. This is backed up with evidence from previous experiments in the Tanis laboratory that have found no additional benefit in paralysis assays from either nutrient in control animals and animals with high B12 and choline content diets, from the *E. coli* strain HB101 (Lam et al., 2021). Those data used strains

expressing Aβ in the body wall muscles, so this comparison suggests that pan-neuronally expressing Aβ strains similarly see no additional benefit from excess supplementation.

When comparing values in the B12 versus the choline trials, the chemotaxes indices in choline trials were lower overall, except for unsupplemented Aβ animals, which had a higher value that that of unsupplemented Aβ animals in the B12 trials. This could suggest some differences in assaying techniques, but the differences are minimal and insignificant so therefore are possibly due to random chance, though it could contribute to the reason why I had no significant difference between supplemented and unsupplemented Aβ animals. Regardless, these data led to me experimenting only with B12 supplementation for the conditioning assay.

I believed that the conditioning assay could give some insight into how Aβ effects memory and learning in the *C. elegans* model. In theory, the animals could ‘learn’ to associate IA, an attractant, with starvation, a state of being to avoid, and ‘remember’ that the molecule is associated with this negative state. Then, healthy animals would not be as attracted to IA, animals demonstrating Aβ-proteotoxicity would still be attracted, and Aβ animals with B12 supplementation would act like the control groups. Four trials of the conditioning assay did not produce significant data, but some interesting trends were exposed. Largely, for each condition, only one data point appeared to be far off from the average, but not always from the same trial. This brings reliability of the protocol into question. By the time conditioning assay trials were completed, the general chemotaxis protocol was largely refined. Most adjustments in creating the final protocol for this assay involved conditioning the animals. Since the isoamyl alcohol used during conditioning is undiluted and the IA on the assay plates is diluted 1:100 in water, it is possible that the treatment is not strong enough to elicit a response from the animals. Although, the same treatment concentration was used to produce significant effects in the normal chemotaxis assay with B12. It is possible that the higher initial concentration produces an inability or lessened ability to sense the chemical because of sensory adaptation from overexposure. This would explain why even in the control animals, conditioning did not significantly alter the CI.

One simple explanation for why these data are not significant is that the starvation state during conditioning was not a large enough deterrent to change IA from an attractant to a repellant. One way to test this theory is to perform conditioning assays with a different chemical instead of IA, which could be a repellant or something more complicated. Repellants such as 2-nonanone and 1-octanol depend on specific sensory neuron types (Troemel et al., 1997), and experiments with these chemicals could give insight into Aβ-proteotoxicity in these different neurons, in both chemotaxis and conditioning assays. A potential roadblock with using octanol is that the neurons involved in sensing this chemical are less sensitive in the absence of food (Chao et al., 2004), so the conditioning starvation state might not produce a large enough 'memory' for CI values to be affected in the assay.

Ethanol initially repels *C. elegans*, but after exposure to the chemical, the animals will begin to tolerate and even develop a preference to ethanol (Lee et al., 2009). Some complications to this might be that previous research in wild-type *C. elegans* has found that pre-exposure of 4 or more hours to ethanol is required to develop ethanol preference. Additionally, the presence of food hastens that rate at which this preference develops (Lee et al., 2009). This implies that ethanol would likely be considered a repellant throughout conditioning and the assay.

One way that the conditioning assay differed from the non-conditioning chemotaxis assay was in the length of time of the assay. Although the animals in the normal chemotaxis assay are scored 60 minutes after plating, animals in all groups of the conditioning assay are scored after 90 minutes, and conditioned animals are exposed to IA for 90 minutes before the assay, in concordance with previous literature on the assay (Fang et al., 2019). Lowering either or both times to 60 minutes might show the same trends that the chemotaxis assay demonstrated. Interestingly, also, the trend that B12 rescues naïve Aβ animals was not replicated in these data, possible due to the time differential. A relatively low amount of sodium azide is put on the assay plates to immobilize the animals in each test quadrant, so it is possible that its efficacy decreases after 60 minutes or that animals are able to move after being initially paralyzed and before the scoring at 90 minutes. The problem with the many different approaches and modifications to this

assay is that set-up takes two weeks and that the assay is resource intensive. Still, the results do not show any significance in memory and learning improvement with B12 supplementation.

Overall, these related projects indicate that B12 has some use in reducing Aβ-proteotoxicity in pan-neuronal Aβ models, and that FAT-5 and FAT-7 acting on 16- and 18-carbon chain fatty acids in fatty acid synthesis are not modulated by B12 availability to change Aβ-proteotoxicity in body wall muscle Aβ models. Further research is needed in both areas to determine if other nutrients are beneficial and if other enzymes in fatty acid synthesis are necessary for healthy function in Aβ animals supplemented with B12.

References

Alzheimer's Association. (2022). 2022 Alzheimer's disease facts and figures. *Alzheimer's & Dementia: The Journal of the Alzheimer's Association*, *18*(4), 700–789. https://doi.org/10.1002/alz.12638

An, Y., Varma, V. R., Varma, S., Casanova, R., Dammer, E., Pletnikova, O., Chia, C. W., Egan, J. M., Ferrucci, L., Troncoso, J., Levey, A. I., Lah, J., Seyfried, N. T., Legido-Quigley, C., O'Brien, R., & Thambisetty, M. (2018). Evidence for brain glucose dysregulation in Alzheimer's disease. *Alzheimer's & Dementia*, *14*(3), 318–329. https://doi.org/10.1016/j.jalz.2017.09.011

Bachurin, S., Bovina, E., & Ustyugov, A. (2017). Drugs in Clinical Trials for Alzheimer's Disease: The Major Trends. *Medicinal Research Reviews*, *37*. https://doi.org/10.1002/med.21434

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics*, *77*(1), 71–94. https://doi.org/10.1093/genetics/77.1.71

Butterfield, D. A., & Halliwell, B. (2019). Oxidative stress, dysfunctional glucose metabolism and Alzheimer disease. *Nature Reviews. Neuroscience*, *20*(3), 148–160. https://doi.org/10.1038/s41583-019-0132-6

Chakravorty, A., Jetto, C. T., & Manjithaya, R. (2019). Dysfunctional Mitochondria and Mitophagy as Drivers of Alzheimer's Disease Pathogenesis. *Frontiers in Aging Neuroscience*, *11*, 311. https://doi.org/10.3389/fnagi.2019.00311

Chao, M. Y., Komatsu, H., Fukuto, H. S., Dionne, H. M., & Hart, A. C. (2004). Feeding status and serotonin rapidly and reversibly modulate a Caenorhabditis elegans chemosensory circuit. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(43), 15512–15517. https://doi.org/10.1073/pnas.0403369101

Chen, G.-F., Xu, T.-H., Yan, Y., Zhou, Y.-R., Jiang, Y., Melcher, K., & Xu, H. E. (2017). Amyloid beta: Structure, biology and structure-based therapeutic development. *Acta Pharmacologica Sinica*, *38*(9), 1205–1235. https://doi.org/10.1038/aps.2017.28

Clare, C. E., Brassington, A. H., Kwong, W. Y., & Sinclair, K. D. (2019). One-Carbon Metabolism: Linking Nutritional Biochemistry to Epigenetic Programming of Long-Term Development. *Annual Review of Animal Biosciences*, *7*(1), 263–287. https://doi.org/10.1146/annurev-animal-020518-115206

Clarke, R., Bennett, D., Parish, S., Lewington, S., Skeaff, M., Eussen, S. J., Lewerin, C., Stott, D. J., Armitage, J., Hankey, G. J., Lonn, E., Spence, J. D., Galan, P., de Groot, L. C., Halsey, J., Dangour, A. D., Collins, R., & Grodstein, F. (2014). Effects of homocysteine lowering with B vitamins on cognitive aging: Meta-analysis of 11 trials with cognitive data on 22,000 individuals1,2,3,4,5. *The American Journal of Clinical Nutrition*, *100*(2), 657–666. https://doi.org/10.3945/ajcn.113.076349

Cline, E. N., Bicca, M. A., Viola, K. L., & Klein, W. L. (2018). The Amyloid-β Oligomer Hypothesis: Beginning of the Third Decade. *Journal of Alzheimer's Disease : JAD*, *64*(s1), S567–S610. https://doi.org/10.3233/JAD-179941

Corsi, A. K., Wightman, B., & Chalfie, M. (2015). A Transparent Window into Biology: A Primer on Caenorhabditis elegans. *Genetics*, *200*(2), 387–407. https://doi.org/10.1534/genetics.115.176099

Cresci, G. A., & Bawden, E. (2015). Gut Microbiome: What We Do and Don't Know. *Nutrition in Clinical Practice : Official Publication of the American Society for Parenteral and Enteral Nutrition*, *30*(6), 734–746. https://doi.org/10.1177/0884533615609899

Deline, M. L., Vrablik, T. L., & Watts, J. L. (2013). Dietary Supplementation of Polyunsaturated Fatty Acids in Caenorhabditis elegans. *Journal of Visualized Experiments: JoVE*, *81*, e50879. https://doi.org/10.3791/50879

Dickinson, D. J., & Goldstein, B. (2016). CRISPR-Based Methods for Caenorhabditis elegans Genome Engineering. *Genetics*, *202*(3), 885–901. https://doi.org/10.1534/genetics.115.182162

Dostal, V., & Link, C. D. (2010). Assaying β-amyloid Toxicity using a Transgenic C. elegans Model. *JoVE*, *44*, e2252. https://doi.org/10.3791/2252

Drake, J., Link, C. D., & Butterfield, D. A. (2003). Oxidative stress precedes fibrillar deposition of Alzheimer's disease amyloid β-peptide (1–42) in a transgenic Caenorhabditis elegans model. *Neurobiology of Aging*, *24*(3), 415–420. https://doi.org/10.1016/S0197-4580(02)00225-7

Fang, E. F., Hou, Y., Palikaras, K., Adriaanse, B. A., Kerr, J. S., Yang, B., Lautrup, S., Hasan-Olive, M. M., Caponio, D., Dan, X., Rocktäschel, P., Croteau, D. L., Akbari, M., Greig, N. H., Fladby, T., Nilsen, H., Cader, M. Z., Mattson, M. P., Tavernarakis, N., & Bohr, V. A. (2019). Mitophagy inhibits amyloid-β and tau pathology and reverses cognitive deficits in models of Alzheimer's disease. *Nature Neuroscience*, *22*(3), Article 3. https://doi.org/10.1038/s41593-018-0332-9

Fong, S., Teo, E., Ng, L. F., Chen, C.-B., Lakshmanan, L. N., Tsoi, S. Y., Moore, P. K., Inoue, T., Halliwell, B., & Gruber, J. (2016). Energy crisis precedes global metabolic failure in a novel Caenorhabditis elegans Alzheimer Disease model. *Scientific Reports*, *6*(1), Article 1. https://doi.org/10.1038/srep33781

Ford, A. H., & Almeida, O. P. (2019). Effect of Vitamin B Supplementation on Cognitive Function in the Elderly: A Systematic Review and Meta-Analysis. *Drugs & Aging*, *36*(5), 419–434. https://doi.org/10.1007/s40266-019-00649-w

Green, R., Allen, L. H., Bjørke-Monsen, A.-L., Brito, A., Guéant, J.-L., Miller, J. W., Molloy, A. M., Nexo, E., Stabler, S., Toh, B.-H., Ueland, P. M., & Yajnik, C. (2017). Vitamin B12 deficiency. *Nature Reviews Disease Primers*, *3*(1), 17040. https://doi.org/10.1038/nrdp.2017.40

Greener, M. (2021). What has Caenorhabditis elegans ever done for us? *Prescriber*, *32*(11–12), 29–32. https://doi.org/10.1002/psb.1955

Iqbal, K., & Grundke-Iqbal, I. (2010). Alzheimer's disease, a multifactorial disorder seeking multitherapies. *Alzheimer's & Dementia : The Journal of the Alzheimer's Association*, *6*(5), 420–424. https://doi.org/10.1016/j.jalz.2010.04.006

Kang, J. H., Cook, N., Manson, J., Buring, J. E., Albert, C. M., & Grodstein, F. (2008). A trial of B vitamins and cognitive function among women at high risk of cardiovascular disease. *The American Journal of Clinical Nutrition*, *88*(6), 1602–1610. https://doi.org/10.3945/ajcn.2008.26404

Kim, H.-M., & Colaiácovo, M. P. (2016). CRISPR-Cas9-Guided Genome Engineering in C. elegans. *Current Protocols in Molecular Biology*, *115*, 31.7.1-31.7.18. https://doi.org/10.1002/cpmb.7

Kivipelto, M., Helkala, E.-L., Laakso, M. P., Hänninen, T., Hallikainen, M., Alhainen, K., Soininen, H., Tuomilehto, J., & Nissinen, A. (2001). Midlife vascular risk factors and Alzheimer's disease in later life: Longitudinal, population based study. *BMJ*, *322*(7300), 1447–1451. https://doi.org/10.1136/bmj.322.7300.1447

Lam, A. B., Kervin, K., & Tanis, J. E. (2021). Vitamin B(12) impacts amyloid beta-induced proteotoxicity by regulating the methionine/S-adenosylmethionine cycle. *Cell Reports*, *36*(13), 109753. https://doi.org/10.1016/j.celrep.2021.109753

Lee, J., Jee, C., & McIntire, S. L. (2009). Ethanol Preference in C. elegans. *Genes, Brain, and Behavior*, *8*(6), 578–585. https://doi.org/10.1111/j.1601-183X.2009.00513.x

Lin, B., Hasegawa, Y., Takane, K., Koibuchi, N., Cao, C., & Kim-Mitsuyama, S. (2016). High-Fat-Diet Intake Enhances Cerebral Amyloid Angiopathy and Cognitive Impairment in a Mouse Model of Alzheimer's Disease, Independently of Metabolic Disorders. *Journal of the American Heart Association*, *5*(6), e003154. https://doi.org/10.1161/JAHA.115.003154

Lin, M. T., & Beal, M. F. (2006). Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature*, *443*(7113), 787–795. https://doi.org/10.1038/nature05292

Long, J. M., & Holtzman, D. M. (2019). Alzheimer Disease: An Update on Pathobiology and Treatment Strategies. *Cell*, *179*(2), 312–339. https://doi.org/10.1016/j.cell.2019.09.001

Lyon, P., Strippoli, V., Fang, B., & Cimmino, L. (2020). B Vitamins and One-Carbon Metabolism: Implications in Human Health and Disease. *Nutrients*, *12*(9). https://doi.org/10.3390/nu12092867

Margie, O., Palmer, C., & Chin-Sang, I. (2013). C. elegans Chemotaxis Assay. *Journal of Visualized Experiments: JoVE*, *74*, 50069. https://doi.org/10.3791/50069

Mohajeri, M. H., Troesch, B., & Weber, P. (2015). Inadequate supply of vitamins and DHA in the elderly: Implications for brain aging and Alzheimer-type dementia. *Nutrition*, *31*(2), 261–275. https://doi.org/10.1016/j.nut.2014.06.016

Morris, M. C. (2009). The role of nutrition in Alzheimer's disease: Epidemiological evidence. *European Journal of Neurology*, *16*(s1), 1–7. https://doi.org/10.1111/j.1468-1331.2009.02735.x

Mullen, G. P., Mathews, E. A., Vu, M. H., Hunter, J. W., Frisby, D. L., Duke, A., Grundahl, K., Osborne, J. D., Crowell, J. A., & Rand, J. B. (2007). Choline transport and de novo choline synthesis support acetylcholine biosynthesis in Caenorhabditis elegans cholinergic neurons. *Genetics*, *177*(1), 195–204. https://doi.org/10.1534/genetics.107.074120

Müller, T., Meyer, H. E., Egensperger, R., & Marcus, K. (2008). The amyloid precursor protein intracellular domain (AICD) as modulator of gene expression, apoptosis, and cytoskeletal dynamics-relevance for Alzheimer's disease. *Progress in Neurobiology*, *85*(4), 393–406. https://doi.org/10.1016/j.pneurobio.2008.05.002

Pagani, L., & Eckert, A. (2011). Amyloid-Beta Interaction with Mitochondria. *International Journal of Alzheimer's Disease*, *2011*, 925050. https://doi.org/10.4061/2011/925050

Rand, J. B. (2007). Acetylcholine. In *WormBook: The Online Review of C. elegans Biology [Internet]*. WormBook. https://www.ncbi.nlm.nih.gov/books/NBK19736/

Reitz, C., & Mayeux, R. (2014). Alzheimer disease: Epidemiology, diagnostic criteria, risk factors and biomarkers. *Biochemical Pharmacology*, *88*(4), 640–651. https://doi.org/10.1016/j.bcp.2013.12.024

Riddle, D. L., Blumenthal, T., Meyer, B. J., & Priess, J. R. (Eds.). (1997). *C. elegans II* (2nd ed.).

Samuel, A., Murthy, V., & Hengartner, M. (2001). Calcium dynamics during fertilization in C. elegans. *BMC Developmental Biology*, *1*, 8. https://doi.org/10.1186/1471-213X-1-8

Scarmeas, N., Stern, Y., Tang, M.-X., Mayeux, R., & Luchsinger, J. A. (2006). Mediterranean diet and risk for Alzheimer's disease. *Annals of Neurology*, *59*(6), 912–921. https://doi.org/10.1002/ana.20854

Singson, A. (2001). Every Sperm Is Sacred: Fertilization in Caenorhabditis elegans. *Developmental Biology*, *230*(2), 101–109. https://doi.org/10.1006/dbio.2000.0118

Singson, A., Mercer, K. B., & L'Hernault, S. W. (1998). The C. elegans spe-9 Gene Encodes a Sperm Transmembrane Protein that Contains EGF-like Repeats and Is Required for Fertilization. *Cell*, *93*(1), 71–79. https://doi.org/10.1016/S0092-8674(00)81147-2

Smith, A. D., Smith, S. M., de Jager, C. A., Whitbread, P., Johnston, C., Agacinski, G., Oulhaj, A., Bradley, K. M., Jacoby, R., & Refsum, H. (2010). Homocysteine-Lowering by B Vitamins Slows the Rate of Accelerated Brain Atrophy in Mild Cognitive Impairment: A Randomized Controlled Trial. *PLOS ONE*, *5*(9), e12244. https://doi.org/10.1371/journal.pone.0012244

Tawfik, A., Elsherbiny, N. M., Zaidi, Y., & Rajpurohit, P. (2021). Homocysteine and Age-Related Central Nervous System Diseases: Role of Inflammation. *International Journal of Molecular Sciences*, *22*(12). https://doi.org/10.3390/ijms22126259

Taylor, M. K., Swerdlow, R. H., & Sullivan, D. K. (2019). Dietary Neuroketotherapeutics for Alzheimer's Disease: An Evidence Update and the Potential Role for Diet Quality. *Nutrients*, *11*(8). https://doi.org/10.3390/nu11081910

Teo, E., Fong, S., Tolwinski, N., & Gruber, J. (2020). Drug synergy as a strategy for compression of morbidity in a Caenorhabditis elegans model of Alzheimer's disease. *GeroScience*, *42*(3), 849–856. https://doi.org/10.1007/s11357-020-00169-1

Teo, E., Lim, S. Y. J., Fong, S., Larbi, A., Wright, G. D., Tolwinski, N., & Gruber, J. (2020). A high throughput drug screening paradigm using transgenic Caenorhabditis elegans model of Alzheimer's disease. *Translational Medicine of Aging*, *4*, 11–21. https://doi.org/10.1016/j.tma.2019.12.002

Teo, E., Ravi, S., Barardo, D., Kim, H.-S., Fong, S., Cazenave-Gassiot, A., Tan, T. Y., Ching, J., Kovalik, J.-P., Wenk, M. R., Gunawan, R., Moore, P. K., Halliwell, B., Tolwinski, N., & Gruber, J. (2019). Metabolic stress is a primary pathogenic event in transgenic Caenorhabditis elegans expressing pan-neuronal human amyloid beta. *ELife*, *8*, e50069. https://doi.org/10.7554/eLife.50069

Thelen, M., & Brown-Borg, H. M. (2020). Does Diet Have a Role in the Treatment of Alzheimer's Disease? *Frontiers in Aging Neuroscience*, *12*, 617071. https://doi.org/10.3389/fnagi.2020.617071

Touroutine, D., & Tanis, J. E. (2020). A Rapid, SuperSelective Method for Detection of Single Nucleotide Variants in Caenorhabditis elegans. *Genetics*, *216*(2), 343–352. https://doi.org/10.1534/genetics.120.303553

Troemel, E. R., Kimmel, B. E., & Bargmann, C. I. (1997). Reprogramming Chemotaxis Responses: Sensory Neurons Define Olfactory Preferences in C. elegans. *Cell*, *91*(2), 161–169. https://doi.org/10.1016/S0092-8674(00)80399-2

van der Veen, J. N., Kennelly, J. P., Wan, S., Vance, J. E., Vance, D. E., & Jacobs, R. L. (2017). The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease. *Membrane Lipid Therapy: Drugs Targeting Biomembranes*, *1859*(9, Part B), 1558–1572. https://doi.org/10.1016/j.bbamem.2017.04.006

Verma, M., Vats, A., & Taneja, V. (2015). Toxic species in amyloid disorders: Oligomers or mature fibrils. *Annals of Indian Academy of Neurology*, *18*(2), 138–145. https://doi.org/10.4103/0972-2327.144284

Walker, A. K., Jacobs, R. L., Watts, J. L., Rottiers, V., Jiang, K., Finnegan, D. M., Shioda, T., Hansen, M., Yang, F., Niebergall, L. J., Vance, D. E., Tzoneva, M., Hart, A. C., & Näär, A. M. (2011). A conserved SREBP-1/phosphatidylcholine feedback circuit regulates lipogenesis in metazoans. *Cell*, *147*(4), 840–852. https://doi.org/10.1016/j.cell.2011.09.045

Watts, J. L. (2016). Using Caenorhabditis elegans to Uncover Conserved Functions of Omega-3 and Omega-6 Fatty Acids. *Journal of Clinical Medicine*, *5*(2). https://doi.org/10.3390/jcm5020019

Watts, J. L., & Ristow, M. (2017). Lipid and Carbohydrate Metabolism in Caenorhabditis elegans. *Genetics*, *207*(2), 413–446. https://doi.org/10.1534/genetics.117.300106

What Are Macronutrients and Micronutrients? (2022, October 5). Cleveland Clinic. https://health.clevelandclinic.org/macronutrients-vs-micronutrients/

Whiley, L., Sen, A., Heaton, J., Proitsi, P., García-Gómez, D., Leung, R., Smith, N., Thambisetty, M., Kloszewska, I., Mecocci, P., Soininen, H., Tsolaki, M., Vellas, B., Lovestone, S., & Legido-Quigley, C. (2014). Evidence of altered phosphatidylcholine metabolism in Alzheimer's disease. *Neurobiology of Aging*, *35*(2), 271–278. https://doi.org/10.1016/j.neurobiolaging.2013.08.001

Yusufov, M., Weyandt, L. L., & Piryatinsky, I. (2017). Alzheimer's disease and diet: A systematic review. *International Journal of Neuroscience*, *127*(2), 161–175. https://doi.org/10.3109/00207454.2016.1155572

Zečić, A., Dhondt, I., & Braeckman, B. P. (2019). The nutritional requirements of Caenorhabditis elegans. *Genes & Nutrition*, *14*(1), 15. https://doi.org/10.1186/s12263-019-0637-7