Effect of temperature, age and lifespan extending interventions on *Caenorhabditis elegans* models of amyloid beta pathology

by

Rachana Subhash Kelkar

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This thesis/dissertation was presented

by

Rachana Subhash Kelkar

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and approved by

Dr. Peter Wipf, Distinguished University Professor, School of Pharmacy

Dr. Arjumand Ghazi, Associate Professor, Department of Pediatrics, School of Medicine

Dr. Barry Gold, Professor, School of Pharmacy

Thesis Advisors: Dr. Peter Wipf, Distinguished University Professor, School of Pharmacy
Dr. Arjumand Ghazi, Associate Professor, Department of Pediatrics, School of Medicine

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Rachana Subhash Kelkar, B. Pharm

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Alzheimer's disease (AD) is an age-related neurodegenerative disease that accounts for 60-70% of all dementia cases worldwide. The hallmark of AD is neuronal death precipitated by accrual of intercellular amyloid (Aβ) aggregates and intracellular neurofibrillary (NFT) Tau tangles. AD risk factors include age, genetics and environment. Despite intense research, the genetic and biochemical underpinnings of AD are poorly understood, and no drugs have been discovered for curing the disease. In this project, we aimed to study the impact of temperature, age and longevity-promoting interventions on two *Caenorhabditis elegans* transgenic strains modeling aspects of Aβ pathophysiology, by expressing full length human (1-42 amino acids) Aβ peptide in muscles or neurons. Since AD is an age-related disease, we first examined how age influenced the dynamics of Aβ-mediated phenotypes, followed by the impact of an additional stress modality-high temperature. As previously reported, worms expressing Aβ in muscles exhibited full body paralysis and mobility defects at high temperatures of 25 °C. Contrarily, the animals expressing Aβ in neurons did not show any paralysis but underwent distinct mobility defects under temperature stress. Surprisingly, we discovered that in both models the extent of pathology was only moderately aggravated by increasing age alone, or upon combining age and temperature stressors. Since aging is the biggest risk factor for AD, we also asked if genetic or chemical interventions known to increase lifespan could impact the phenotypes of the worm Aβ models. We found that a known lifespan-extending drug, promethazine•HCl, significantly delayed the onset of

paralysis and mobility defects in the Aβ (muscle) model on day 1 of the animal. Similarly, we found that two known lifespan extending transcription factors, DAF-16 and NHR-49, also played an important role in alleviating Aβ phenotypes on day 1 and influenced the ability of promethazine•HCl to retard Aβ pathology. Overall, in characterizing an *in vivo* worm platform for identifying drugs and genes that impact AD, we have delineated the interlinked effects of age, temperature and genetic environment on Aβ pathology.

Keywords: Alzheimer's disease, *C. elegans*, animal model, promethazine, lifespan extending drug, genetics, *daf-16, nhr-49, glp-1*

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1.0 Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disease that accounts for 60-70% of all cases of dementia[1, 2] worldwide and is responsible for enormous social and economic impacts on the lives of the patients and caregivers[3]. Aging is the biggest risk factor for the development of the common, sporadic form of AD. AD affects around 5 million Americans[4] at an approximate cost of $100 billion per year.[5] It is estimated that the number of AD patients will reach over 14 million by the year 2050 if there are no further developments in the therapy or preventions of this disease[6, 7]. Despite intense research, the genetic and biochemical pathways that lead to AD are poorly understood and no drugs have been discovered for the cure of this disease. The present classes of drugs that are available for treatment are the acetylcholine esterase (AChE) inhibitors and N-methyl-D-aspartate (NMDA) receptor antagonists[2, 8, 9]. These drugs only provide symptomatic relief in mild to moderate AD patients and hence the root cause is not taken care of[9]. It is the need of the hour to develop drugs that have disease modifying or preventive properties. This poses a huge challenge due to lack of approved therapeutic targets and inadequate validated animal models[10].

Mechanistic molecular-genetic studies and drug screens on AD mouse models[11, 12] are extremely expensive and time consuming since mice live for over 2 years. Hence, moving towards methods and models that are short-lived and genetically amenable is an important avenue for unraveling AD biology and for rapid, high throughput screening (HTS) of potential therapeutics. *In vivo* models such as worms and flies can be especially valuable in terms of drug biology and drug mechanism of action studies.

1.1 **Alzheimer's disease pathology**

AD is mainly characterized by dementia. It begins with Mild Cognitive Impairment (MCI) that includes failure of memory and slowly becomes severe, which includes increase in confusion, poor judgement, language disturbance, visual complaints and agitation[13]. 95% of AD is late-onset and is prevalent in the age groups of 60-65 years and above[14]. The remaining 5% of AD occurs in the age group less than 60 years. The hallmark of AD is neuronal death precipitated by accrual of intercellular amyloid (Aβ) aggregates and intracellular neurofibrillary (NFT) Tau tangles, and therapeutic development focuses on minimizing the aggregation of these proteins in the neurons.

The diagnosis of the disease usually includes neuropathologic findings of β-amyloid plaques, intraneuronal neurofibrillary tangles (tau protein) and amyloid angiopathy[14]. The main molecular basis of AD is defects in protein homeostasis which lead to aggregation of misfolded proteins in the neurons. Two dominant theories or hypotheses have been proposed to explain AD mechanisms. These are the amyloid and the tau hypotheses[15].

The amyloid hypothesis involves a series of events that includes the process and secretion of the amyloid precursor protein (APP) and the imbalance caused between the production and the clearance of amyloid-β, which is an important factor in the development of AD[16]. Amyloid-β is a protein that is extremely resistant to protein degradation[17, 18]. The protein consists of two isoforms, 1-42 being the most common[19]. It is also hydrophobic and highly toxic[20]. Due to its structural configuration, this protein tends to fold into β-pleated sheets,[21] and because of this it has a higher tendency to aggregate and form the core of amyloid plaques[22-24] (Fig. 1).

Tau protein aggregation is a secondary pathogenic event that causes subsequent neurodegeneration[25]. There has been evidence of amyloid-β inducing tau alterations by promoting phosphorylation and cytoplasmic and dendritic translocation linked neurodegeneration[26, 27]. The function of Tau protein is to stabilize microtubules that provide support for axonal transport and neuronal growth.[28-30]
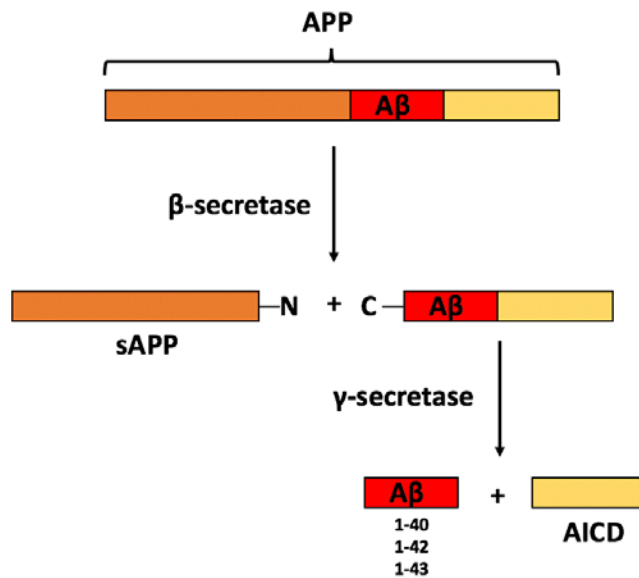
APP: transmembrane amyloid precursor protein
Aβ: amyloid beta
sAPP: soluble APP N-terminal fragment
AICD: APP intracellular domain
1-40,1-42,1-43: variable lengths of Aβ protein

Figure 1: Pathway depicting the functions of amyloid-β and the role of APP

The amyloid precursor protein is a transmembrane protein that is cleaved by the enzyme β-secretase into soluble APP and the remaining transmembrane part. The transmembrane part of the protein is cleaved by γ-secretase into amyloid-β and AICD. This enzyme can cleave Aβ protein in three different ways, at the 40th, 42nd and 43rd amino acid. The cleavage at the 42nd amino acid generates the most toxic fragments.

Recently, the amyloid hypothesis and its involvement in AD have been challenged[31]. Alternative hypotheses suggest that the main factor causing the neurodegeneration may be the tau aggregates that form first. Due to the different structural conformations of these aggregates, the neurons are unable to function normally, leading to neuronal damage[31]. APP (amyloid precursor protein) has been found to trigger tauopathy[32]. Many preventative drugs that have shown positive results in mice models have gone further into clinical trials but almost all of them have failed the clinical studies. Most of these drugs generally fail the phase 3[33] clinical. Importantly in spite of treating the amyloid plaques, the pathology of the disease did not improve in some clinical trials[34]. The debate regarding primary underlying cause for AD notwithstanding, the involvement of Aβ and Tau in AD remains clear. However, our understanding of the cellular and molecular underpinnings of this disease is still poor, and a treatment/drug remains elusive.

1.2 **Risk factors of AD**

There are two different forms of AD: sporadic and familial[4, 35, 36]. The familial form of AD has been reported to result from mutations in at least three of the major genes. On the contrary, the sporadic form of AD can develop due to other genetic and environmental changes. The disease is further categorized into early-onset AD (EOAD) and late-onset AD (LOAD)[37-39]. EOAD accounts for approximately 1-6% of all the cases and roughly spans between the age group of 30-65 years[4, 40]. On the other hand, age is the biggest risk factor for LOAD which is the most common form of AD. The development of LOAD is common in the age groups of 60-65 years or older[4, 41-43]. Both LOAD and EOAD have their association in the familial form of AD, but approximately 60% of the EOAD have multiple cases of AD in their family[4]. The

sporadic form of AD is also the most common form of AD. The reasons behind developing a sporadic form of AD are unclear and involve a complex combination of genes, environmental factors and lifestyle changes. On the other hand, the familial form of AD involves mutations in known specific genes and also is the cause of EOAD[35, 44, 45].

The major risk-factors of AD are age[46], genetics and environmental changes. The prevailing belief, supported strongly by experimental evidence, is that with increasing age there is an accumulation of the above-mentioned amyloid plaques and neurofibrillary tau tangles, and these bodies lead to neuronal death and loss of connectivity precipitating AD symptoms. It is also generally acknowledged that the causative cellular and molecular changes of AD predate the disease symptoms by years, possibly decades[2]. Since aging is the biggest risk factor for AD, one attractive avenue to delay/prevent AD is to delay aging itself, and hence this is a prominent area of current biomedical research[47].

As mentioned above, other important risk factors for AD include genetic mutations and environmental and lifestyle changes. The genetic factors include mutations in genes that lie on the pathway to the production and degradation of the amyloid-β[48, 49]. The three most important gene mutations include *APP* (amyloid precursor protein), *PSEN I* (presenilin 1) and *PSEN II* (presenilin 2)[4, 50, 51]. *APP* is a gene that codes for a cell surface protein that is cleaved by enzymes called secretases[4]. *PSEN1* codes for the protein PSEN1 and this protein is a part of the complex γ-secretase enzyme that is involved in cleaving the APP[52]. *PSEN 2* is a gene that codes for the protein PSEN2 which is also required for precise cleaving the APP [53]. Mutations in any of these genes have been shown to increase the risk to cause/ develop the disease[54-57]. Another genetic component involved in the disease is the presence of the ε4 allele of the gene, *APOE*. *APOE* codes for Apolipoprotein E, a class of enzymes involved in the metabolism of fats in the

body. Studies show that the presence of the APOE(ε4) allele significantly increases the risk of a person to develop AD[58].

Besides age and genetics, environment and lifestyle have important roles in the development of AD. People who have multiple health conditions are predisposed to developing AD compared to healthy individuals[59-61]. In particular, individuals with obesity[61] or diabetes,[62] and metabolic syndrome have increased susceptibility to development of AD, as do ones with cardio-vascular disease (CVD) [2, 63].

1.3 **The *Caenorhabditis elegans* model**

Caenorhabditis elegans (*C. elegans*) is a free-living, microscopic nematode. The adults are 1 millimeter long and the newly hatched larvae are 0.25 millimeters long. They are usually observed under the dissecting microscope or compound microscope. *C. elegans* are maintained and grown on petri dishes with agar. These worms feed on bacteria, and in the lab are fed *Escherichia coli (E. coli)* strain OP50. *C. elegans* is a transparent animal and hence it is easy to look through the body of the animal and observe the various organs inside the animal using Nomarski (differential interference contrast, DIC) optics[64]. Fluorescent reporters (Fig. 2[64]) can be tagged to different proteins that are expressed in the worm. This makes it easier to study developmental defects, screen for proteins in vivo, and study the expression of proteins under certain conditions[64]. Importantly, the worm genome is compact and highly amenable to genetic manipulation. *C. elegans* exists as a self-fertilizing hermaphrodite (Fig. 3[64]), meaning both the male and the female gonad are present in the animal. Males do exist, but at a rate of less than 0.2%. It takes around 3 days at 25 °C for the animal to transform from being an egg to an egg-laying

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adult. A single animal can give rise to thousands of small worms, potentially sparing an entire population.
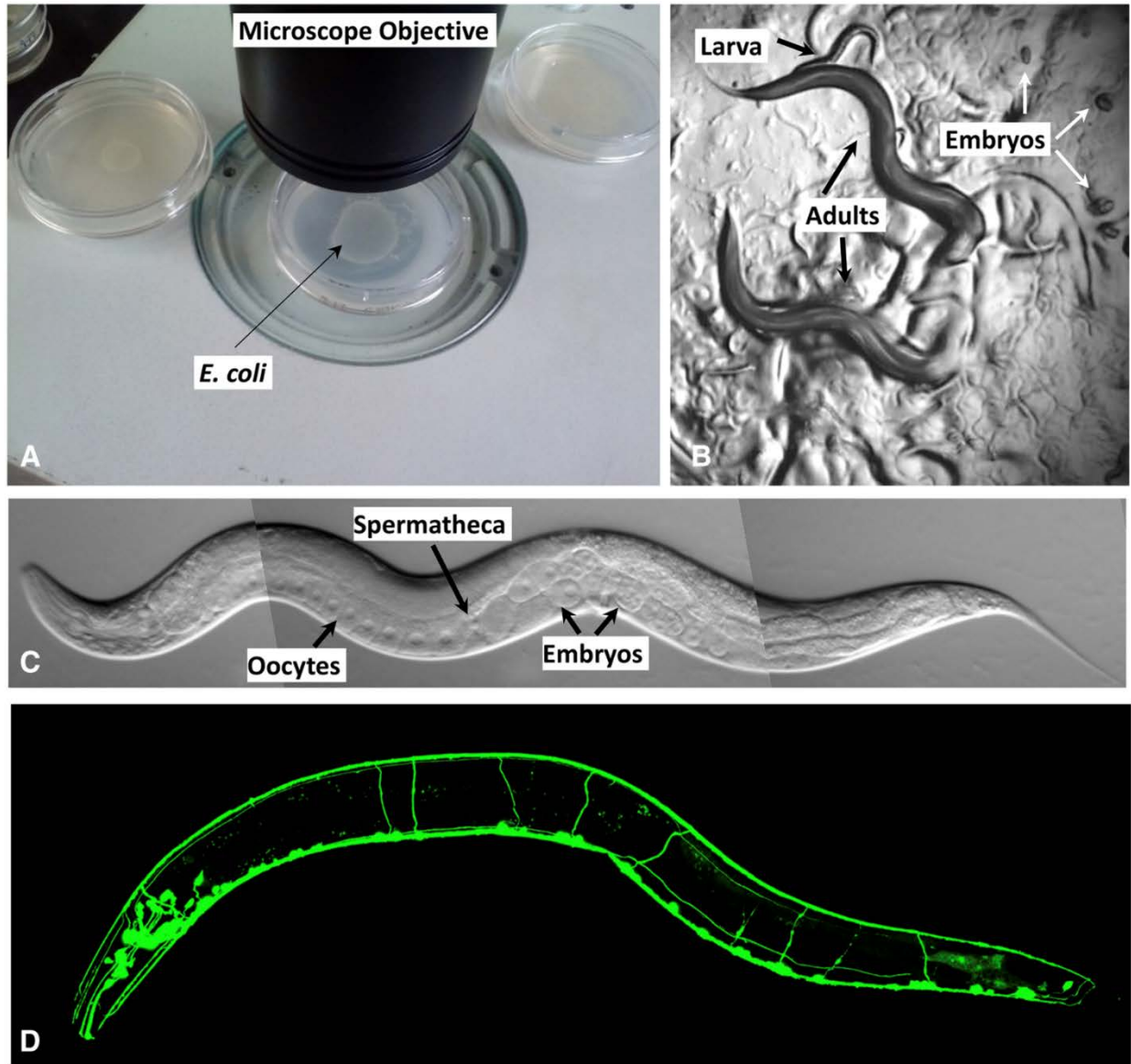
Figure 2: *Caenorhabditis elegans*

A. An image of an agar petri plate seeded with *E. coli* OP50 bacteria used for growing worms placed under a dissecting microscope.

B. Adult worms, embryos and larva shown under the dissecting microscope.

C. Image of a hermaphrodite under a compound micrscope. The spermatheca, oocytes and embryos are clearly visible (labelled) as are the intestine and pharynx (not labelled).

D. Image of a worm with neurons expressing GFP (green fluorescent protein).

(Reproduced with permission from [64])
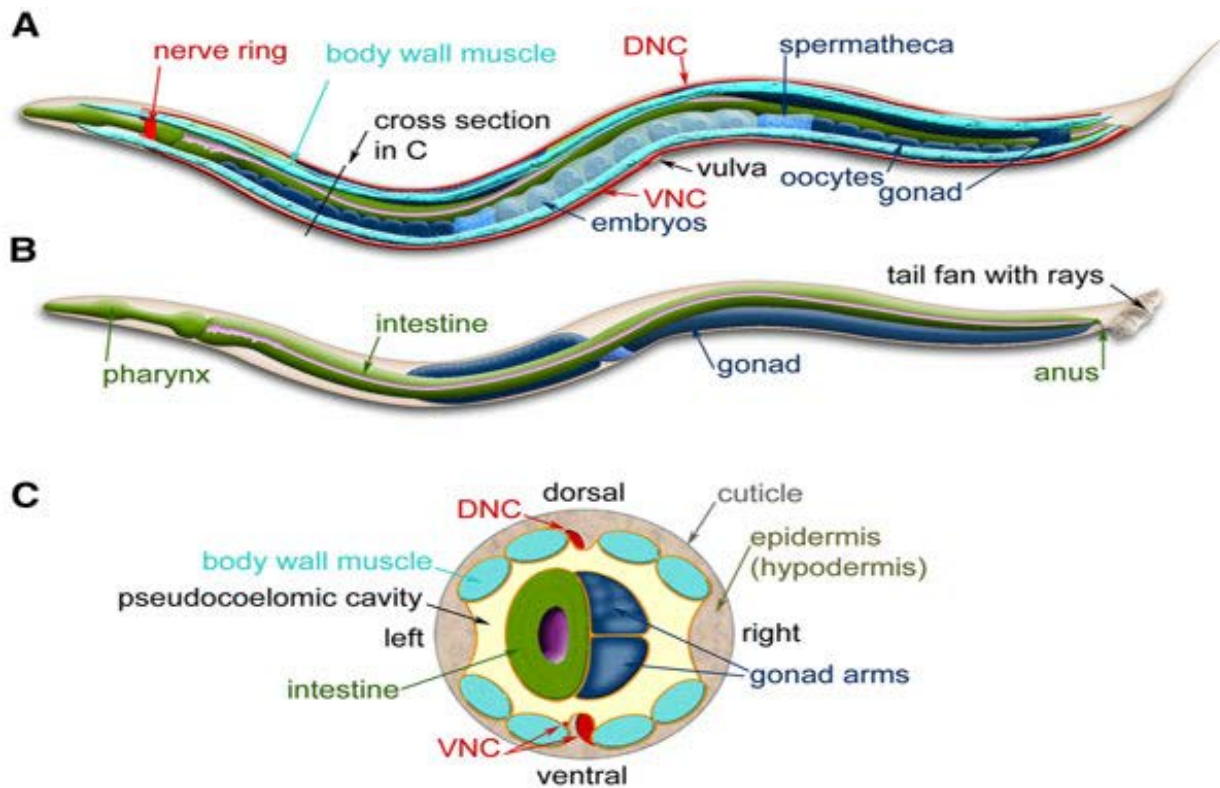
Figure 3: Anatomy of *C. elegans*

    A.   **Lateral section of an adult hermaphrodite**

    B.   **Male C. elegans. The dorsal nerve chord and the ventral nerve chord run along the entire length of the animal from the nerve ring. The pharynx and the intestine of the animal are visible in green.**

    C.   **Cross section through anterior region of the *C. elegans* hermaphrodite with different tissues highlighted.**

(Reproduced with permission from [64])

The life cycle (Fig. 4[64]) of *C. elegans* begins with the hermaphrodite laying down internally fertilized eggs. Eggs hatch into L1 (Larval 1) stage, further followed by L2, L3 and L4 developmental stage. L4 is the last stage of larval life and is characterized by precise physical and physiological landmarks. In particular, the vulva and uterus show very stereotypical features that

are easily recognizable and can be used to isolate age-matched L4 larva. In an early L4 larva, the uterus appears 'half-moon shaped', whereas, late L4 larva show a characteristic 'Christmas-tree-shaped' uterus and an everted vulva[64]. This is not only an important developmental landmark but also significant experimentally. In most cases, experiments targeting adults (such as chemical treatment or RNAi) are commenced at the L4 larval stage. A hermaphrodite can be distinguished from a male by its wider girth and tapered tail compared to a slimmer girth and fan shaped tail of the male.
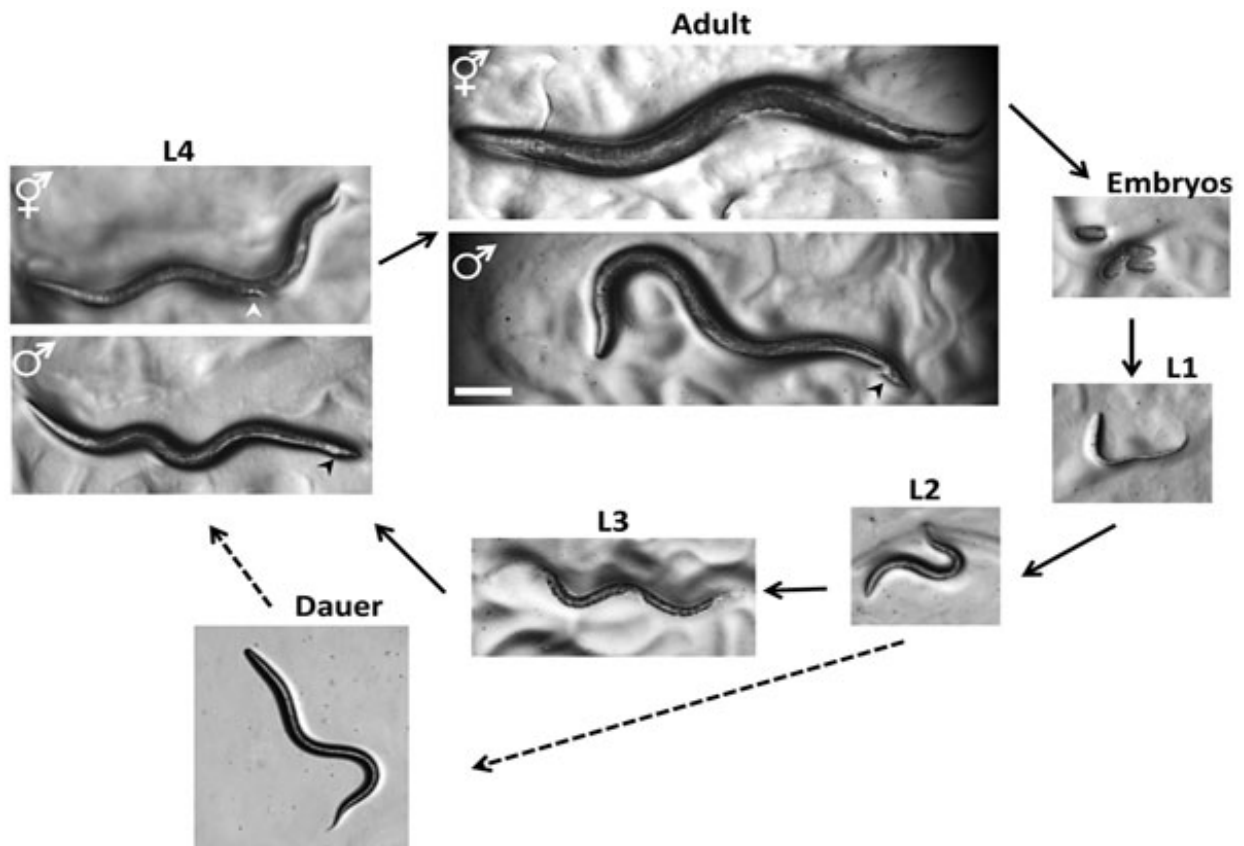
Figure 4: Life cycle of *C. elegans*

The life cycle starts with the adult worm laying eggs that hatch into L1 larvae, followed by development of L1 into L2, L3, L4 and adults.

(Reproduced with permission from [64])

C. elegans was the first multi-cellular organism whose entire genome was sequenced[65]. One important reason why *C. elegans* makes an ideal organism to study human diseases is the presence of highly conserved biochemical pathways between humans and worms[66, 67]. 60-80% of the genes are predicted to have direct or indirect homologs in the human genome and around 40% of worm genes are associated with human diseases[2]. RNAi interference (RNAi) is extremely easy to undertake in worms and this has made it possible to inactivate individual genes and examine the consequences thereof on different parameters. The genetic and molecular amenability, along with high homology with human genes, have led to the development of numerous worms model of human disease [68-71]. These mutations help mimic the disease state in worms and are also easy to score based on different phenotype.

The nervous system of the worm consists of a total of 302 neurons out of which 282 neurons belong to the large somatic system and the remaining 20 are a part of the small pharyngeal nervous system. The neural networks that make up the entire nervous system of the animal have been visualized[72, 73]. The function of each neuron can be linked to each neural circuit. Although the nervous system of the worm is simple, the functions that are carried out by each neuron are complex and closely related to the functions carried out by human neurons[21, 73, 74]. The ease of maintenance, together with rapid life cycle, genetic homology and transparency makes *C. elegans* an excellent model system to study neurodegenerative and other diseases.

1.4 *Caenorhabditis elegans* models of Alzheimer's Disease

C. elegans has become a well-utilized platform for different neurodegenerative diseases., due to the reasons described above. Although worms cannot recapitulate the obvious symptoms of

most neurodegenerative diseases such as cognitive abnormalities and neuronal death, transgenic strains expressing human disease proteins [such as the polyglutamine tracts (polyQ) that underlie Huntington's Disease or alpha-synuclein involved in Parkinson's Disease] exhibit cellular, molecular and physiological consequences that represent disease pathology. For instance, in worms expressing long (35+) stretches of polyQ, exposure to high temperatures causes dramatic polyQ aggregation (as seen in disease conditions) and subsequent paralysis of the animal. These phenotypes have proven to be very valuable assays to study the influence of different genes, drugs and environmental conditions on molecular aspects of HD pathology. Similarly, transgenic strains expressing Aβ (amyloid β) and Tau peptides in various C. elegans tissues have been generated, and are being used to dissect Aβ and Tau biology. In particular, expressing Aβ in muscles (strain GMC101) has been shown to make the animal susceptible to full-body paralysis upon exposure to high temperatures and this has proven to be a simple readout for Aβ pathology in many studies[75]. Similarly, Aβ expression in neurons (strain CL2355)[72] has been shown to reduce mobility in worms. Thus, while it is important to recognize that these strains cannot, and do not, simulate the human AD itself, they provide a simple biological readouts and hence are valuable platforms to identify genes, environmental factors and drugs that can influence cellular and molecular biology of Aβ and Tau.

The two strains used in this study as models of Aβ biology are as follows:

1. GMC101- *dvIs100 [unc-54p::A-beta-1-42::unc-54 3'-UTR + mtl-2p::GFP]* and

2. CL2355- *dvIs50 [pCL45 (snb-1::Abeta 1-42::3' UTR(long) + mtl-2::GFP[72]].*

The rationale for choosing these strains was to test potential anti-AD drugs and interventions on models where physiological phenotypes have resulted from Aβ expression in different tissues. The GMC101 strain was used more widely in our project since it showed a

stronger and 'easier-to-score' phenotype[19, 76]. GMC101 L4-staged worms when exposed to a temperature of 25 °C underwent full-body paralysis. This paralysis was due to the aggregation of the human Aβ peptide in the muscles of the animal[76]. The CL2355 (Aβ neuron) strain on the other hand expresses the protein in the neurons of the animal and exhibits strong mobility defects[77]. This includes defects of thrashing in liquid, defects in chemotaxis and associated learning. Defects of thrashing in liquid and paralysis were the two phenotypic changes that were measured in these mutants for our experiments. In this project, we hypothesized that these *C. elegans* strains could be useful platform to dissect the individual and complex roles of genetic and environmental risk factors influencing Aβ pathology, and for screening drugs with anti-AD potential.

2.0 Material and Methods

2.1 Nematode strains and maintenance

The temperature sensitive mutants GMC101[27, 76] and CL2355[27, 77] along with the control strain Bristol N2 were used in all the experiments. All strains were obtained from the *Caenorhabditis* Genetics Centre (CGC), University of Minnesota. The strain AGP276 was generated by crossing the GMC101 (*Aβ* [muscle]) males with the CF1880[78] (*daf-16;glp-1*) double mutant hermaphrodites. The double mutant of *Aβ (muscle);nhr*-49 and the triple mutant *Aβ (muscle);nhr*-49*;glp-1* were generated by crossing GMC101 (*Aβ* [muscle]) males with *nhr-49;glp-1* double mutant hermaphrodites. All strains were raised and maintained at 15 °C, and exposed to 25 °C for the expression of the mutation. All animals were age-synchronized in the experiment by picking L4 (larval stage 4) worms. Age-matching with bleach was avoided to eliminate any bleach related stress on the animals. Animals were grown on nutrient growth medium (NGM) plates seeded with live *E. coli* OP50 bacteria.

2.2 Solvents and chemicals

All solvents, including DMSO (for the drug) and ethanol (for the cholesterol solution), were reagent grade (Fisher Scientific). Around 600 mg of the chemical, promethazine •HCl (CAS no. 58-33-3) was obtained from the Wipf Lab, Department of Chemistry, Chevron Science Center, University of Pittsburgh, Pittsburgh, Pennsylvania. M9 buffer solution was prepared by mixing 3

g of KH_2PO_4, 6 g of Na_2HPO_4 and 5 g NaCl in 1 L of deionized water. The mixture was sterilized using the autoclave on cycle 12 for liquids (60 mins at 121 °C at 15 psi). 1 mL of 1 M $MgSO_4$ was added to the cooled M9 solution to prevent degradation of $MgSO_4$.

10x PCR (100 mM Tris, 500 mM KCl, 15 mM MgCl2 pH 8.3 dNTP mix: 25 mM/each Primers: 5-10 uM Taq Polymerase: approx 5U/ul) buffer was diluted to 1x using pure water and 20 mg/mL of proteinase K was added to this mix to prepare the worm lysis buffer.

2.3 **Preparation of NGM plates**

The standard protocol[79] was used to prepare nutrient growth medium (NGM) plates. For the preparation of 1 L of media, 3 g of NaCl, 17 g of agar, 2.5 g of bacto peptone and deionized water was used to generate a volume to 1 L. This media was autoclaved on cycle 12 for liquids (60 mins at 121 °C at 15 psi). The media was cooled in a water bath (55 °C) for 15-30 min. 25 mL of 1 M KPO_4 buffer pH 6.0 (108.3 g of KH_2PO_4, 35.6 g K_2HPO_4, deionized water to 1 L), 1 mL of each 1 M $MgSO_4$, 1 M $CaCl_2$ and 5 mg/mL of cholesterol in ethanol was added to the media later to prevent degradation of these compounds at higher temperature. 10 mL of media was poured in each 10 cm petri plate and around 6 mL of media was poured to a 3 cm petri plate. All experiments were performed on 10 cm plates except for thrashing which was performed on 3 cm plates. The plates were seeded with *E. coli* OP50 bacteria which was inoculated in LB (lysogeny broth) and incubated in a 36 °C shaker for longer than 18h. 100 μ L of OP50 culture was seeded on 10 cm plates and 50 μ L of culture was seeded on 3 cm plates.

2.4 **Preparation of drug plates**

The drug was given to the worm through their food source. The plates were made fresh 2 4 days before the experiment in a similar way as described above. The plates were poured and allowed to dry overnight. The next day, these plates were seeded with OP50 (alone), OP50 with 0.4 % DMSO (as control), OP50 with 100 uM of the drug promethazine•HCl[80] in 0.4% DMSO and OP50 with 500 uM of drug in 0.4% DMSO. The seeded plates were kept overnight to dry and were used for the experiment the next day. A stock solution of 25 uM (to give a final concentration of 100 uM) and 125 uM (to give a final concentration of 500 uM) in DMSO (0.4%) was used to prepare a final solution of the drug in 100 uL of bacteria (seeded on each plate). One stock solution was used throughout a set of experiment and new solution was prepared with each new trial.

2.5 **Thrashing assay**

L4 staged worms were picked from a population that was maintained at 15 °C and transferred to new plates and kept at 25 °C for 24 h/ 48 h/ 96 h depending on the type of experiment[81]. Five separate 5-cm mini plates were marked with numbers 1-5 and filled with 1 mL M9 solution. Worm plates were taken out of the incubator after 24 h/ 48 h/ 96 h and 20 worms were transferred to another unseeded mini plate to remove bacteria from the worms. After 5-10 min one worm was transferred onto one mini plate and after 5 min the stopwatch was started for 30 secs. Using a counter, the number of thrashes/ body bends was counted every time the worm moved in the opposite direction of the previous body bend.

2.5.1 Thrashing assay to check effect of temperature on mutant model

Unless otherwise specified, all the strains were maintained at 15 °C. L4 (larval stage 4) animals were picked and subjected to temperature stress by transferring the animals to 25 °C. The animals were exposed to 25 °C (for 24, 48 and 96 h) and scored for thrashing as day 1, 2, and day 4 animals (Fig. 6).
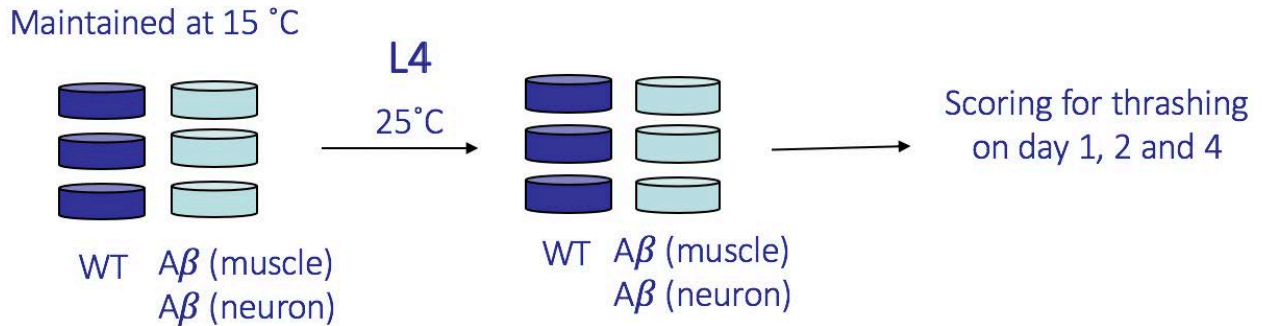
Figure 5: Schematic representation of experimental Scheme 2.5.1

2.5.2 Thrashing assay to check the effect of age on mutant model

L4 staged animals were picked and maintained at 15 °C and 20 °C without subjecting them to temperature stress until day 10 of their lives. They were scored for thrashing on day 2, 4, 6, 8, and 10 (Fig. 7).
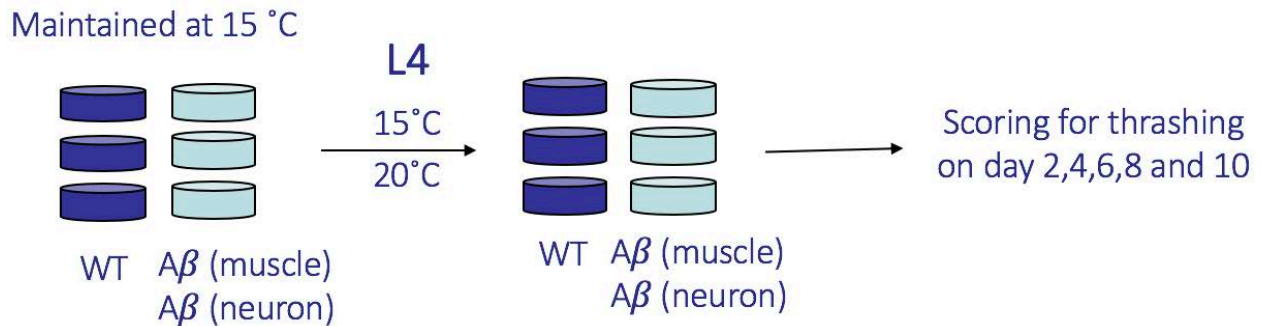
Figure 6: Schematic representation of experimental Scheme 2.5.2

2.5.3 **Thrashing assay to check the combined effect of temperature and age**

The worms were maintained at 15 °C. L4 stage worms were picked and maintained at 15 °C. Animals were subjected to temperature stress on day 2, 4, 6, 8 and 10 for 24 h at 25 °C. They were scored for thrashing after 24 h (Fig. 8).
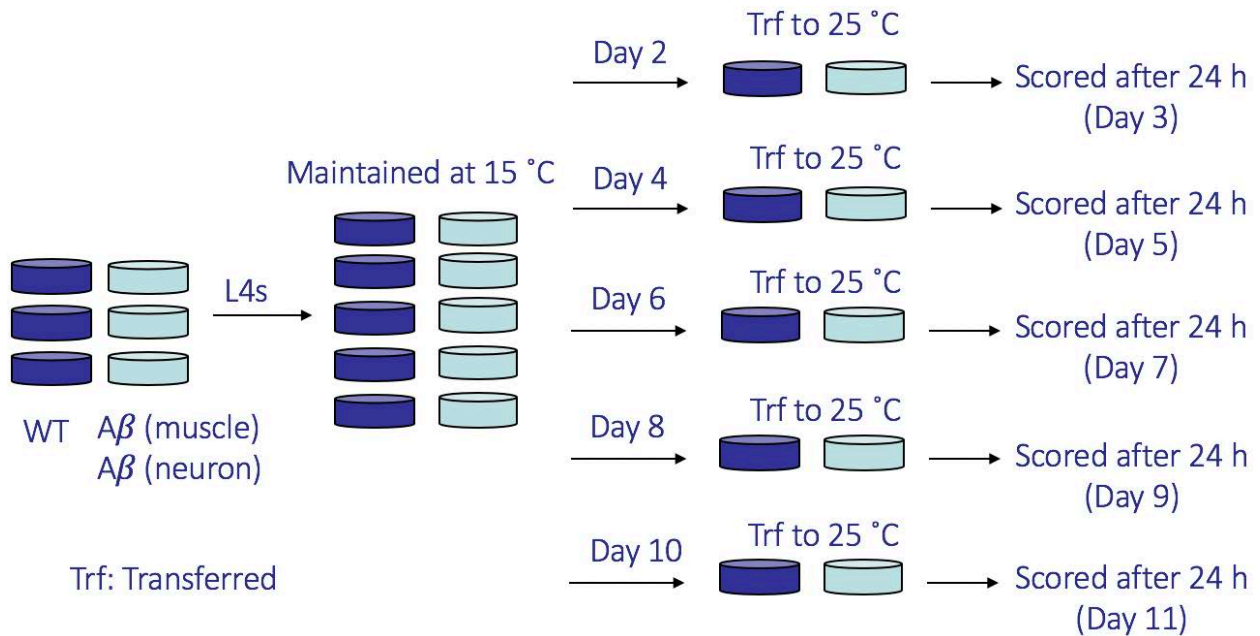
Figure 7: Schematic representation of experimental Scheme 2.5.3

2.6 **Paralysis assay**

Similar to the thrashing assay, L4 staged worms were picked and maintained at designated temperature. The mutant worms were exposed to temperature stress by warming them to 25 °C for varied duration depending on the need of the experiment. Post exposure to temperature stress, the worms were scored as paralyzed or not paralyzed[81]. To confirm paralysis, the worms were touched with a standard sized pick. If the body of the worm moved, it was scored as not paralyzed.

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To confirm paralysis, the worm was picked and transferred to another spot on the same plate. If the entire body of the worm did not move, but the head moved, the worm was still scored as paralyzed.

2.7 Lifespan assay

Wild type animals were transferred as L4's on 10 cm treated and untreated NGM plates and according to standard lifespan protocol[82], they were maintained at 20 °C throughout their lifespan. Dead, missing and alive worms were counted everyday/ every alternate day until all the worms on the plates were dead. All bagged worms (a phenomenon where the eggs hatch internally within the parent and the larvae try to escape the parent)[83-85] were counted towards missing. The worms were transferred every two days onto new plates to avoid clustering of the worms on the plates[82]. A slightly modified lifespan assay was performed wherein the animals were administered with the drug from day 8 of their life. The worms were still maintained at 20 °C throughout their lifespan.

2.8 Genetic crosses

To investigate the effect of different pro-longevity genes on the AD model of *C. elegans* we wanted to introduce loss-of-function mutations in key longevity-promoting genes into that GMC101 strains expressing Aβ protein in the muscles. We selected genes that we and others had previously found to extend lifespan in the worms. *daf-16[86]* encodes a transcription factor DAF-

16 (homolog of human protein FOXO3A) that plays a major role in the insulin/insulin like growth factory signaling (IIS) pathway[87]. This transcription factor up-regulates the expression of innumerable genes that contribute to the extension of the lifespan of the animal. In *C. elegans*, germline loss has been shown to dramatically increase lifespan and stress resistance, dependent on the presence of *daf-16[86, 87]*. The germline-less longevity can be re-created using a temperature sensitive mutant in the gene *glp-1*[88] that is essential for germ cell proliferation. To test if this longevity-extending intervention also conferred resistance to Aß pathology, we introduced the *Aß* transgene into the *glp-1* background.

Besides age and genetics, other major AD risk factors are environmental influences and lifestyle. Obesity increases the predisposition to developing AD as does diabetes and dyslipidemia[61]. We used mutants for a gene, nhr-49, that encodes the worm homolog of PPARα (peroxisome proliferator-activated receptor alpha), a key regulator of lipid metabolism[89-91], to examine the metabolic influences on Aß pathology. We crossed the mutant expressing the Aß protein in the muscle of the worm into different strains devoid of the following above-mentioned genes: *daf-16, glp-1, nhr-49*. A brief summary of the cross protocol is given below.

2.8.1 GMC101 (Aß [muscle]) crossed into CF1880 (*daf-16; glp-1*)

The GMC101 strain was crossed into the CF1880 (*daf-16; glp-1*) strain[92]. A GMC101 male plate was prepared by transferring around 10 hermaphrodites onto 3 mini plates each and heat shocked in the 33 °C water bath for 4-6 h[93]. The plates were then kept at 20 °C and allowed to grow. Males were picked. Around 3 plates were set up with 2 plates of a 4:1 and 1 plate of a 5:2 ratio of males to hermaphrodites. The plates were maintained at 20 °C. The worms were allowed to grow and 10 L4 hermaphrodites were setup, 1 worm per mini plate, to allow the individual

worm to propagate. It was made sure that the worms were green in color under the GFP fluorescent scope which ensured that the cross was successful (since only the males were GFP tagged). The F1 progeny was allowed to grow and 80 plates of F2 progeny were setup (1 worm per mini plate) and allowed to reproduce to generate progeny. The parents were transferred to another plate and the animals were transferred to 25 °C to confirm for *glp-1* mutation. When the parents were found to give rise to enough (at least 30 eggs on each plate) progeny, they were lysed for the confirmation of *daf-16* through PCR. The new mutant was named as AGP276 and frozen in the worm library.

Figure 8: Cross of GMC101 into AGP22

2.8.1 GMC101 (Aß [muscle]) crossed into AGP22 (*nhr-49; glp-1*)

The GMC101 strain was crossed into the AGP22 (*nhr-49; glp-1*) strain. A GMC101 male plate was prepared by transferring around 10 hermaphrodites onto 3 mini plates each and heat shocked in the 33 °C water bath for 4-6 h[93]. The plates were then kept at 20 °C and allowed to grow. Males were picked. It was made sure that the worms were green in color under the GFP fluorescent scope which ensured that the cross was successful (since the males were GFP tagged). Around 3 plates were set up with 2 plates of a 4:1 and 1 plate of a 5:2 ratio of males to hermaphrodites. The plates were maintained at 20 °C. A second cross was performed by picking males on the cross plate and picking L4 stage hermaphrodites of the AGP22. The ratio was 10 males to 3 hermaphrodites. The worms were allowed to grow and 10 L4 hermaphrodites were setup, 1 worm per mini plate, to allow the individual worm to propagate. The F2 progeny was allowed to grow and 80 plates of F3 progeny were setup (1 worm per mini plate) and allowed to reproduce to generate progeny. The parents were transferred to another plate and the animals were transferred to 25 °C to confirm for *glp-1* mutation. When the parents were found to give rise to enough (at least 30 eggs on each plate) progeny, they were lysed for the confirmation of *nhr-49* through PCR.

Scheme to cross GMC101 (*Aβ muscle*) into AGP22 *nhr-49;glp-1(-)* genetic background

P0 (Cross #1)	GMC101 $\frac{A\beta}{A\beta}$ ♂	X	AGP22 $\frac{nhr\text{-}49(-);glp\text{-}1(-)}{nhr\text{-}49(-);glp\text{-}1(-)}$ ♀

F1 (Cross #2)	$\frac{A\beta}{+}$; $\frac{nhr\text{-}49(-);glp\text{-}1(-)}{+}$ ♂	X	$\frac{A\beta}{+}$; $\frac{nhr\text{-}49(-);glp\text{-}1(-)}{+}$ ♀

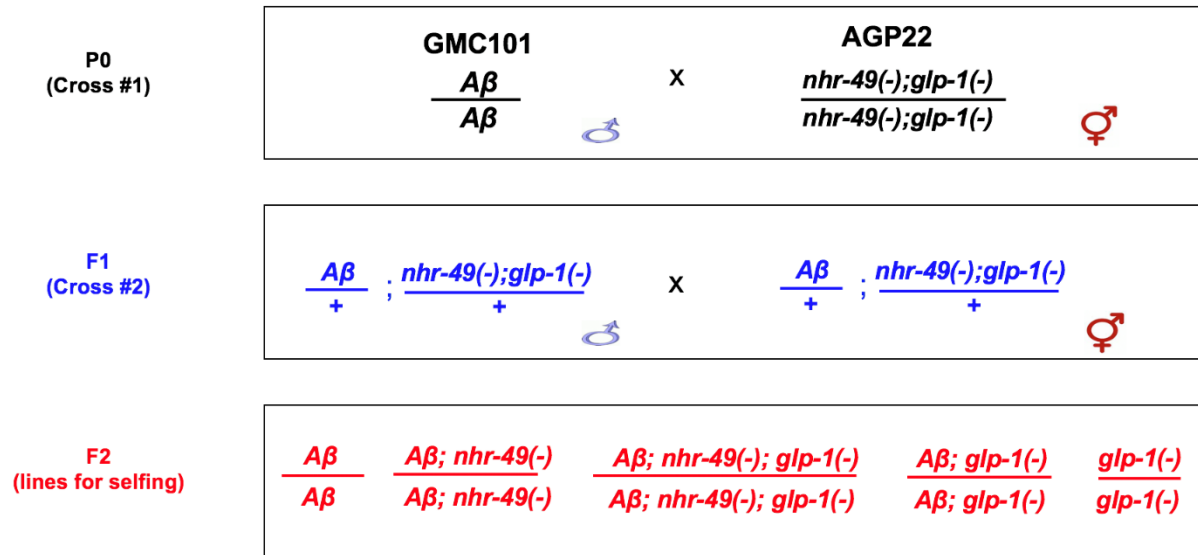| F2 (lines for selfing)	$\frac{A\beta}{A\beta}$	$\frac{A\beta;\ nhr\text{-}49(-)}{A\beta;\ nhr\text{-}49(-)}$	$\frac{A\beta;\ nhr\text{-}49(-);\ glp\text{-}1(-)}{A\beta;\ nhr\text{-}49(-);\ glp\text{-}1(-)}$	$\frac{A\beta;\ glp\text{-}1(-)}{A\beta;\ glp\text{-}1(-)}$	$\frac{glp\text{-}1(-)}{glp\text{-}1(-)}$
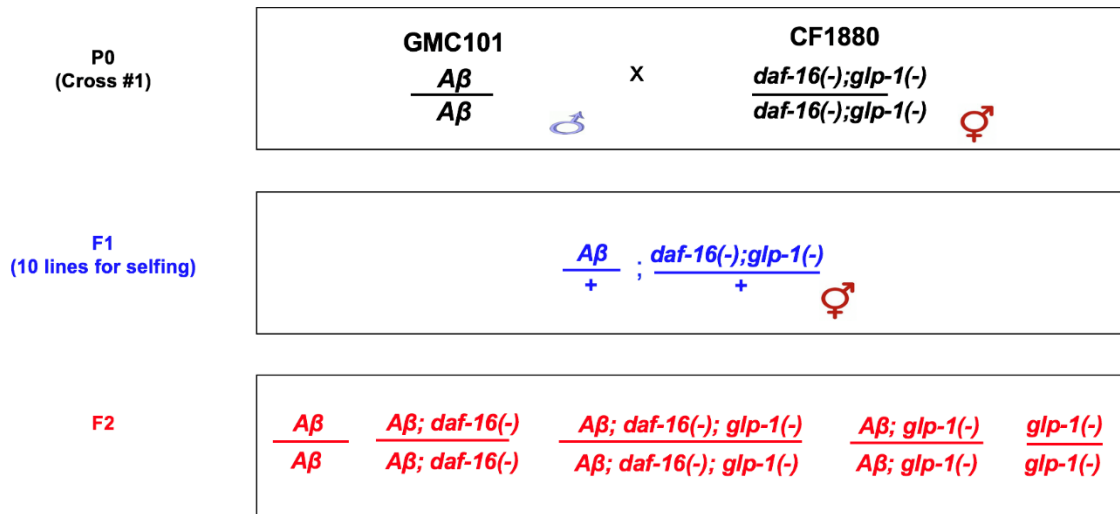
Confirm with GFP the Aβ mutation: 100% animals on the plate should have;
Confirm with sterility test for *glp-1*: 100% of progeny should be sterile
nhr-49 PCR: mothers should be homozygous for *nhr-49*

Figure 9: Cross of GMC101 into AGP22

2.8.2 Molecular diagnostic tests for daf-16 and nhr-49

The *glp-1(e2144ts)* mutation causes a temperature-sensitive loss of fertility, and this sterility was used as the diagnostic test to identify glp-1 mutation in crosses. The *daf-16(mu86)* null mutant we used here carried a 10980 bp deletion[94] in the *daf-16* genes that resulted in a complete loss of protein function. The *nhr-49(nr2041)* mutation was a 893 bp[94] deletion that eliminated part of intron 3, all of exon 4 and some of exon 5 from the K20C3.6c transcript[95].

To confirm the mutation in crosses, PCR (polymerase chain reaction) was used to amplify the piece of DNA of interest. To carry out PCR confirmation the worms were first lysed[96] using 5 uL of worm lysis solution (995 uL of 1X PCR buffer with 5 uL of proteinase K) for normal PCR (1 parent adult worm/tube) and 10 uL of worm lysis buffer (around ~5 adult worms/tube) for

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population PCR. The tubes were kept at -80 °C for 15-30 min. The worm lysis program cycle (60° 60', 95° 15', 4° hold) was used on PCR.

5 uL of this lysate solution was added to 20 uL of the PCR mix (Table 1). Post worm lysis, GoTaq and primers were added specific to *daf-16* and the PCR cycle (94° 3', [94° 45", 55° 30", 72° 1'30"]x29, 72° 10', 4° hold) was carried out. PCR mix differed from gene to gene.

The PCR mix for *daf-16* was as follows:

Table 1: Formula of PCR mix for *daf-16* mutation

INGREDIENT	AMOUNT/TUBE (uL)
GoTaq	12.5
KL4	1.5
KL5	1.5
KL15	1.5
KL32	1.5
ddH$_2$O	1.5
Worm lysate	5
TOTAL	25

For *nhr-49* confirmation using PCR, the same steps were followed except for the program used in the PCR machine. The program for *nhr*-49 (95° 2', [95° 30", 62° 30", 72° 1']x40, 72° 10", 4° hold) was used on the PCR machine. This program was optimized by Dr. Ghazi.

The PCR mix for *nhr-49* was as follows:

Table 2: Formula of PCR mix for *nhr-49* mutation

INGREDIENT	AMOUNT/TUBE (uL)
GoTaq	12.5
Mut F	0.5
R_1	0.5
R_2	0.5
ddH_2O	6
Worm lysate	5
TOTAL	25

The GoTaq contained all the primers and the nucleotides. The strength of GoTaq was 2X hence half the actual amount of it was used in the PCR mix.

Agarose gel electrophoresis was carried out on PCR products and it was used to separate DNA fragments. 1 g of agar was dissolved in 100 mL of buffer solution. 15 uL of ethidium bromide was used to tag DNA. This chemical intercalates in DNA[97] and allows to visualize different bands in the image reader. The gel was analyzed in a Biorad Gel Doc EZ Imager and on the Image Lab 6.0 software.
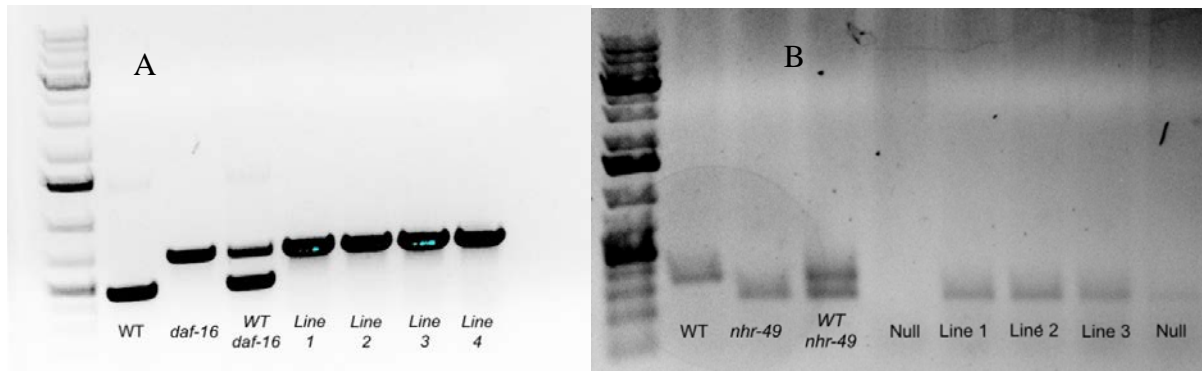
Figure 10: Mutant confirmation using gel electrophoresis post PCR

A. Inverted images of confirmation of *daf-16* mutation. The first three lanes are control with WT (437 bp) in the first, *daf-16* (657 bp) mutant in the second and mix of both in the third. There were four lines of the *daf-16* mutant obtained. All the lines were homozygous for the *daf-16* mutation.

B. Confimation of *nhr-49* (250 bp) mutation with first three lanes having the WT (350 bp) control and mix of both. 3 lines were obainted that were homozygous for the mutation.

2.9 **Statistical analysis**

Statistical analysis was conducted in GraphPad Prism version 8.0.1 (145). Unless otherwise stated, data were analyzed using unpaired t-test. All data were reported and shown as mean±SD (Standard Deviation). A survival curve for lifespan assay was plotted in the OASIS 2 software. For the lifespan assay, Kaplan-Meier statistics with Log-Rank Test were utilized.

3.0 Results

3.1 Characterization of the AD mutant models of *C. elegans*

The GMC101 and CL2355 strains' phenotypes have been well documented. However, how these features are impacted by age or temperature stress (or both) has not been investigated in detail. We characterized the mutant models by the effects of age and temperature individually and then by examining them together, since both are important risk factors for AD. The rationale behind testing the temperature effect was to check if temperature stress would cause protein aggregation in the muscles and neurons of these mutants, respectively. Temperature stress was found to disrupt the protein homeostasis in the animals which would in turn cause the misfolding of the protein causing it to aggregate and bring about phenotypic changes in the animal. We also wanted to test if combining age and temperature stress might result in aggravation of Aß pathology.

3.1.1 Effect of temperature on paralysis and mobility features

We observed that exposing the GMC101 worms showed a significant decline in the number of thrashes at a very early stage in the animals' life. The thrash number was seen to be significantly lower in GMC101 animals compared to the WT animal on day 2 (Fig. 10-A). Similarly, a significant decline in the thrash number was observed in CL2355 strain on day 2 and further decrease was seen on day 4 (Fig. 10-B). This was in accordance with previous reports, and led us

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to conclude that temperature stress aggravates Aβ pathology whether expressed in the neurons or in the muscle.
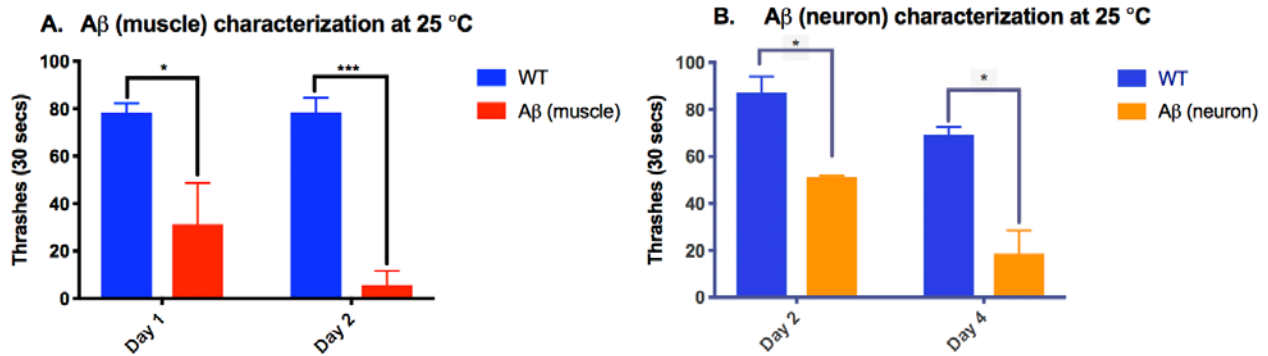
Figure 11: Effect of temperature on mobility features

A. Effect of temperature stress on thrashing assay of GMC101 worms

B. Effect of temperature stress on thrashing assay of CL2355 worms

Experiments were performed in two independent trials with n=20 per trial. Student's unpaired t-test was used to carry out statistical analysis. * indicates p<0.05, *** p<0.0001

With respect to paralysis, around 60% of the GMC101 worms were found to be paralyzed under temperature stress of 25 °C. On the contrary, the CL2355 worms were not seen to be paralyzed at all. Hence, aggregation of the protein within the neurons does not appear to show a strong phenotype as opposed to when the aggregation took place in the muscle. However, this strain has been reported to exhibit behavioral deficits including defects in to associated learning of the association of food and odorant [77].

3.1.2 **Effect of age on paralysis and mobility features**

Since age is the single biggest risk factor for AD, we hypothesized that the GMC101 strain would exhibit pathology as animals aged, without exposure to high temperature, or that temperature stress would accelerate paralysis in older animals. However, surprisingly this was not the case. Older animals that were maintained at lower temperatures throughout their lives (15 °C and 20 °C) showed mobility comparable to wild-type control animals. GMC101 did not show any further decrease in the number of thrashes as old animals at 15 °C and 20 °C compared to WT animals. The thrashing number in GMC101 strain was consistently lower than that of the WT at every age, suggesting that the presence of the Aβ protein induces aggregation that is sufficient to cause loss of mobility (Fig. 10-A and 10-C), and increasing age did not aggravate this dramatically.
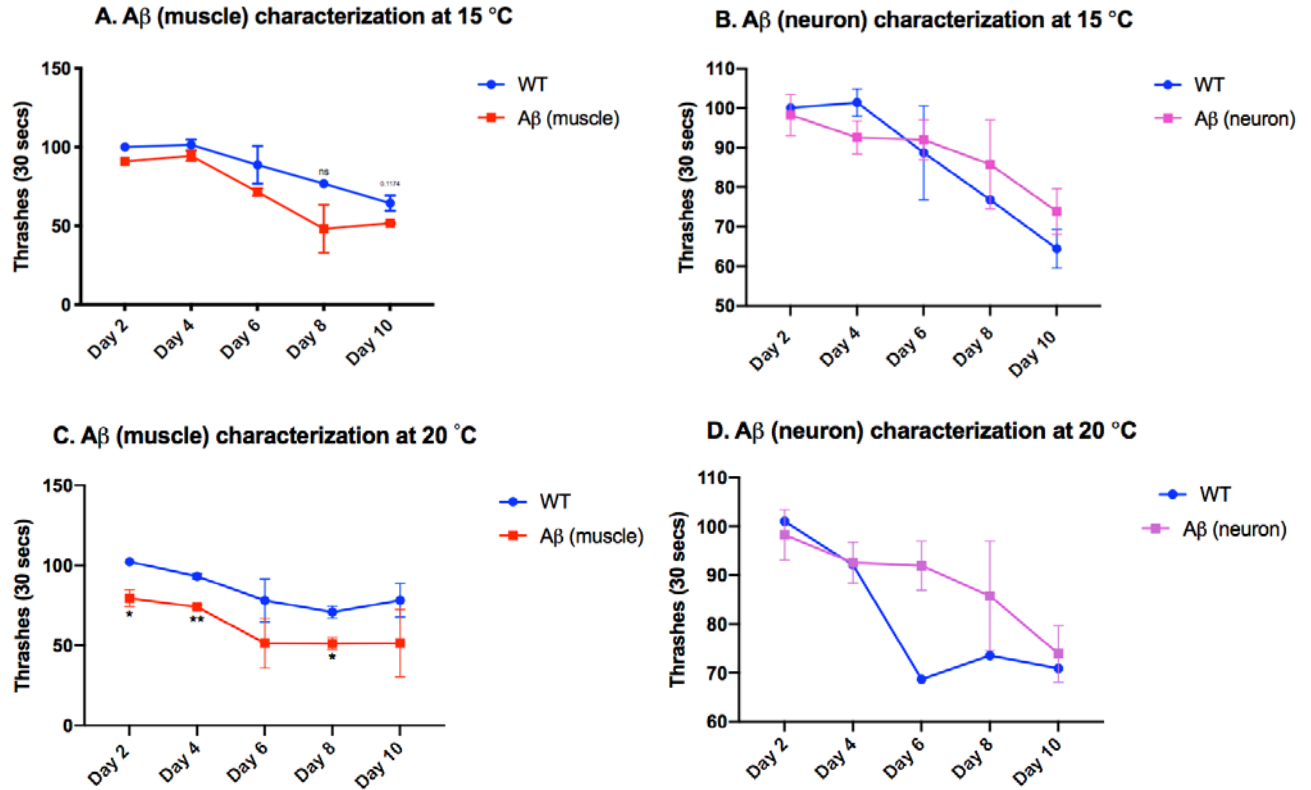
Figure 12: Effect of age on mobility features

A. and C. Effect of thrashing on GMC101 worms when maintained at 15 °C and 25 °C respectively

B. and D. Effect of thrashing on CL2355 worms maintained at 15 °C and 25 °C respectively

Experiments were performed in two independent trials with n=20 per trial. Student's unpaired t-test was used to carry out statistical analysis. * indicates $p < 0.5$, ** $p < 0.01$

Surprisingly, the CL2355 strain showed mobility comparable to that of WT in young animals (day 2 and day 4) adults but showed improved mobility on days 6, 8, and 10 (Fig. 10-B and 10-B). Thus, it appears that while the presence of the Aβ peptide itself in muscles or neurons is sufficient to destabilize mobility, the effects are not dramatically impacted as the animal ages. It was possible that longer exposures to high temperatures are required to bring about stronger phenotypic changes in the mutants.

3.1.1 **Effect of temperature and age on paralysis and mobility features**

We wanted to analyze the combined effect of temperature and age on these Alzheimer's mutants and wanted to see if age and temperature stress together caused a further degradation in the mutant phenotype compared to the WT animals. Hence, we exposed the animals to temperature stress at different stages of the worms' life. Worms were exposed to 25 °C as day 2, 4, 6, 8 and 10 and scored for thrashing 24 h post higher temperature exposure. The animals were maintained at 15 °C before exposing them to higher temperature of 25 °C.

As Alzheimer's is an age-related disease[46], we expected that the older animals might show defects in their mobility much earlier than the younger animals when exposed to temperature stress. But to our surprise, we observed that there was no difference between the thrashes in older (day 4 and 6) and much older animals (day 8 and 10).

GMC101 worms showed a decrease in the number of thrashes post day 9 but the decrease was not significant (Fig. 12-A). Similar to GMC101 worms, CL2355 showed also a decrease in the number of thrashes post day 7, but the difference was not significant (Fig. 12-B).

A. Temporal characterization of Aβ (muscle) at 25 °C

B. Temporal Characterization of Aβ (neuron) at 25°C
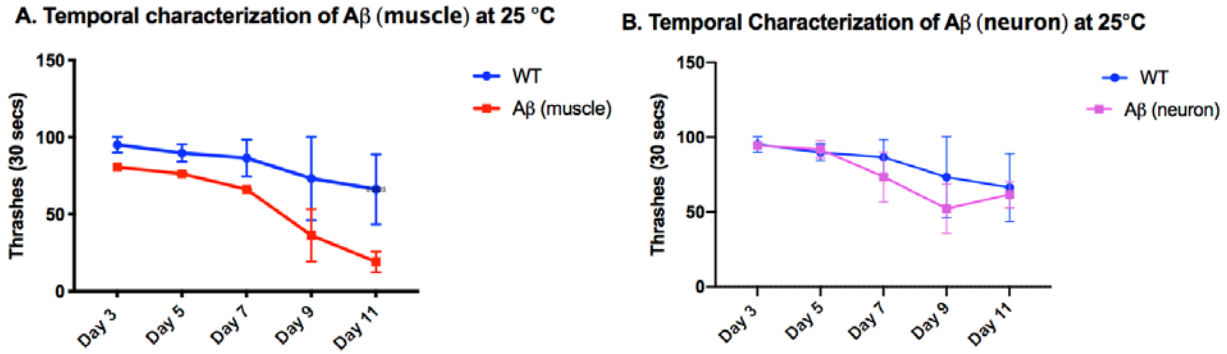
Figure 13: Effect of temperature and age on mobility features

A. Effects of GMC101 worms on thrashing when older animals are exposed to higher temperatures (25 °C)

B. Effects of CL2355 worm son thrashing when older animals are exposed to higher temperature (25 °C)

Experiments were performed in two independent trials with n=20 per trial. Student's unpaired t-test was used to carry out statistical analysis.

We also exposed older animals (on day 2, day 4 and day 6) to temperature stress (25 °C) and we were surprised to see that none of them were paralyzed after 48 h. This might suggest that the sole presence of Aβ in the worm has no phenotypic effect on the animal. Hence, exposing older animals to higher temperature of 25 °C had no significant effect on the phenotype, suggesting that this model of *C. elegans* might not be an accurate representation of the Aβ pathology. The results were consistent with the previous conclusion (Fig. 11), where there was no decrease in the number of thrashes in the older animals as compared to the younger ones.

A. Paralysis of Aβ (muscle) as older animals
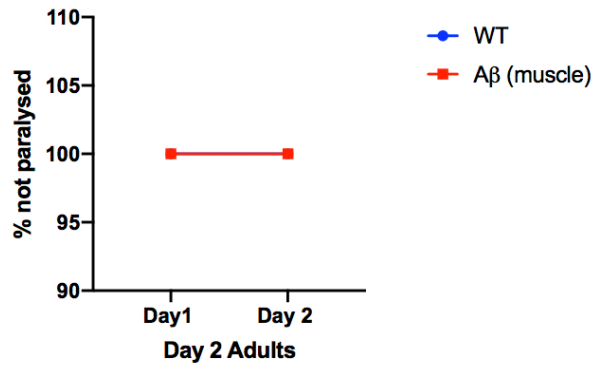
B. Paralysis of Aβ (muscle) as older animals

C. Paralysis of Aβ (muscle) as older animals

Figure 14: Effect of old age and temperature on paralysis of GMC101 animals

The worms were transferred to higher temperature on day 2 (A), day 4 (B), day 6 (C) and none of them were

seeme to be paralyzed. The experiments were performed in three independent trials with n=20. Student's

unpaired t-test was used to carry out statistical analysis.

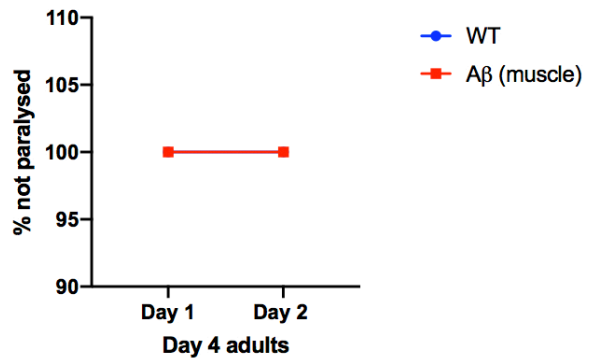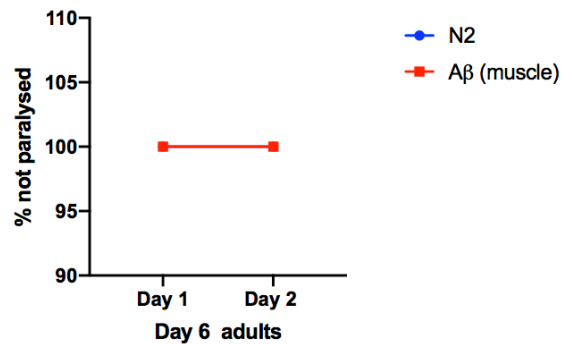## 3.2 Screening of drugs on the mutant models

3.2.1 Screening of promethazine•HCl on wild type *C. elegans*

A previous study[80] conducted a high throughput screening (HTS) of drugs that were already marketed for different disease conditions to check their lifespan-extending properties in *C. elegans*. The categories of drugs included various anti-histamines, calcium channel blockers, anti-biotics, adrenergics, hormones, potassium channel blockers, etc. One of the lifespan-extending drugs identified in this study was promethazine•HCl (CAS NO.: 58-33-3), an anti-histamine indicated for allergic conditions and for motion sickness. In the published report, it was found to extend the lifespan of *C. elegans* by 32%[80]. Since AD is an age-related disorder, we asked if increasing lifespan might delay the onset of the disease or prevent it and tested the effect of this drug on the Aß pathology in Alzheimer's mutants.

We reproduced the lifespan assay on the WT animals on solid media[82] and on different concentrations (33 uM and 100 uM in one experiment and 100 uM and 500 uM in the other) of the drug (Fig. 14 and 15). It was observed that there was no significant difference in the extension of lifespan when the worms were treated with a lower concentration of drug (33 uM) but there was a significant increase in the longevity of the animal when treated with a higher concentration of the drug (100 uM) (Fig. 14). Since AD is detected later in life and as treatment begins thereafter, we wanted to check if
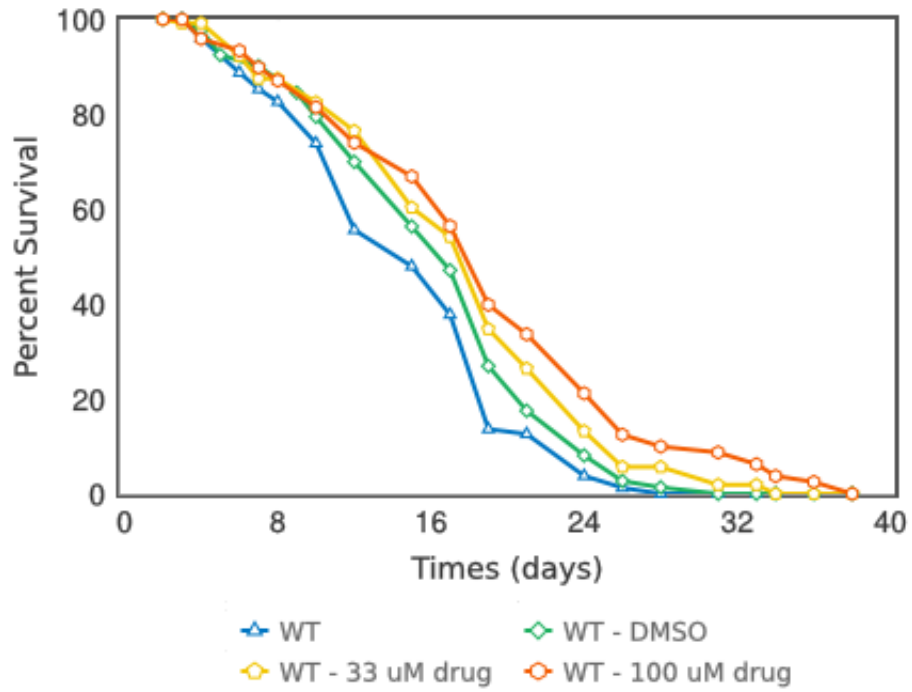
Figure 15: Survival curve of WT (N2) animals at different concentration of promethazine•HCl

This figure is a representation of one trial with n=100 per condition. Kaplan Meier (log-rank test) statistics were used to carry out statistical analysis for lifespan assays. P 500 uM vs N2 DMSO 0.0085**
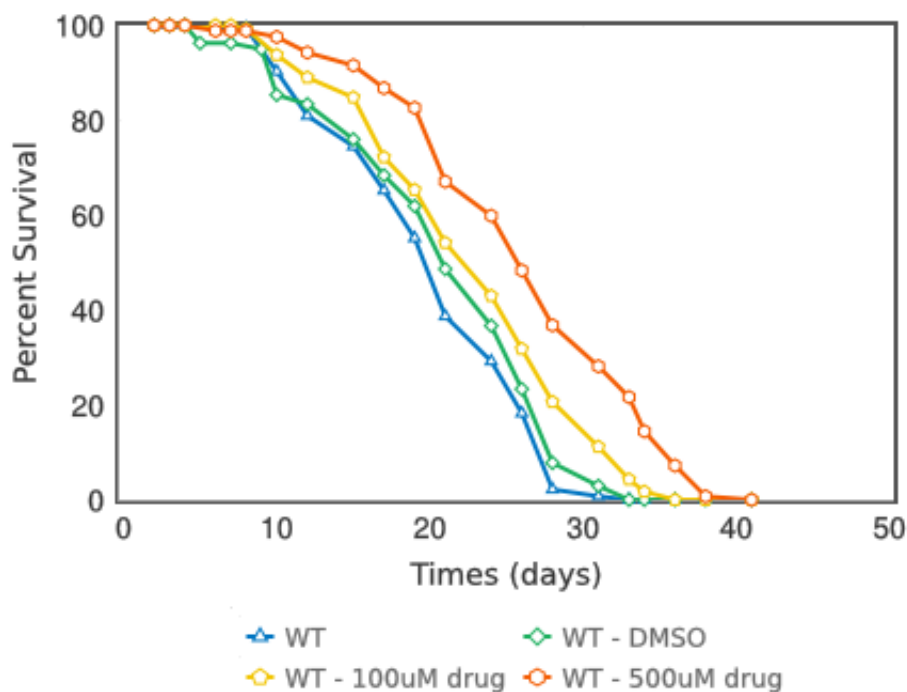
Figure 16: Survival Curve of WT (N2) animals at different concentration of promethazine•HCl

3.2.2 Screening of promethazine•HCl on GMC101 worms

First, we confirmed that promethazine•HCl extended the lifespan of WT *C. elegans*. Next, we tested to see if this lifespan extending drug improved Aß pathology. The lower drug concentration of 33 uM had no significant difference compared to the solvent-only DMSO control and hence the concentration of the drug was increased to 500 uM. The drug was administered to 20 worms on each plate, and the worms were scored for thrashing and paralysis.

3.2.2.1 **Effect on thrashing assay**

There was no significant difference in the number of thrashes of WT and the GMC101 worms. It could be observed that the effect was higher in the GMC101 worms treated with the

drug compared to the WT worms treated with the drug (Fig. 16-A and 16-B) when compared to their respective DMSO controls. There was no significant difference between the treatment group and the DMSO control group in the WT animals. Although the thrash number was higher in the treated GMC101 worms compared to the DMSO control, the effect was insignificant.

Figure 17: Effect of promethazine•HCl on thrashing of GMC101

A. Thrashing of WT worms when treated with promethazine•HCl

B. Thrashing of GMC101 worms when treated with promethazine•HCl

Experiments were performed as two independent trials with n=20. Student's unpaired t-test was used to carry out statistical analysis. * indicates p<0.05

3.2.2.2 Effect on paralysis assay

The GMC101 worms were treated with two different concentration of drugs – 100 uM and 500 uM. These concentrations were kept constant from the previous survival assay. The worms treated with the higher concentration of the drug (500 uM) showed a significant decrease in the percentage of paralyzed worms. There was no significance seen in the worms that were treated with a lower concentration (100 uM) of the drug (Fig. 17). This demonstrated that the effect was dose dependent. The comparison was done between the treated and the DMSO control.

methazine•HCl on Aβ (muscle)
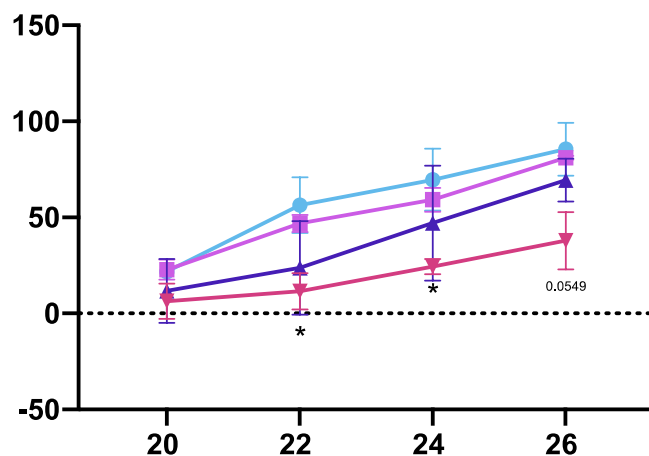
Figure 18: Effect of promethazine•HCl on paralysis of GMC101

The mutant was treated with two different concentration of the drug. Experiments were performed as two independent trials with n=10 per conditions. Student's unpaired t-test was used to carry out statistical analysis. * indicates p<0.05

3.2.3 Screening of promethazine•HCl on CL2355 worms

Promethazine•HCl was found to have a positive effect on the muscle Aβ model of *C. elegans*. Since, in AD, there are amyloid plaques that are observed in the neurons, we wanted to observe the effect of the drug in other mutant that expressed the Aß protein in the neurons of the worm. The only phenotype that we focused on was the defect in thrashing.

3.2.3.1 **Effect on thrashing assay**

The worms were treated with two different drug concentrations of drug – 100 uM and 500 uM. Overall, the CL2355 mutants on drug had a higher thrash number compared to the WT

animals. Hence, the drug promethazine•HCl improved the Aß pathology. The thrash number for

CL2355 worms was significantly higher for the treated group compared to the DMSO control.

(Fig. 18-A and 18-B). Surprisingly, the drug was also seen to have some effect on the WT animal.
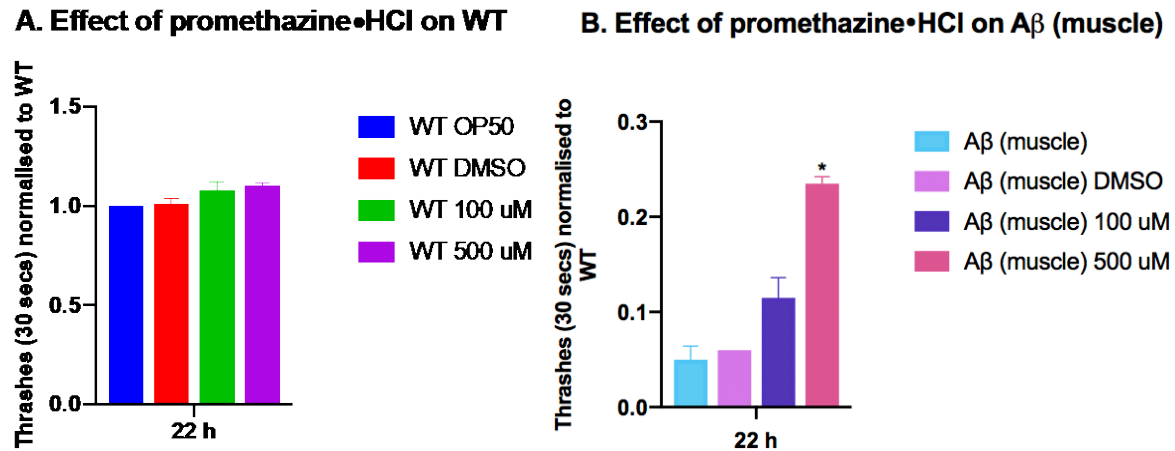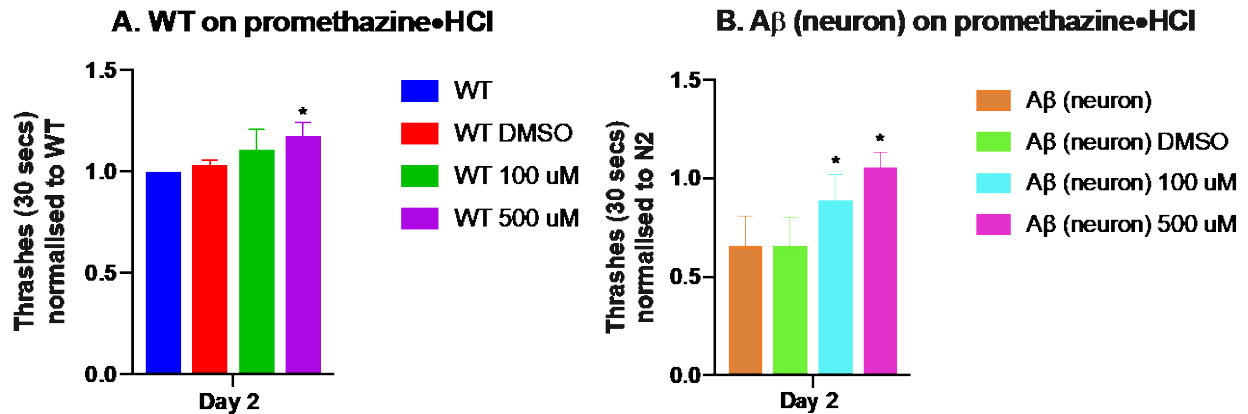

Figure 19: Effect of promethazine•HCl on thrashing of CL2355

A. **Thrashing of WT worms when treated with promethazine•HCl**

B. **Thrashing of GMC101 worms when treated with promethazine•HCl**

Experiments were performed as two independent trials with n=10 in each group. Student's unpaired t-test was used to carry out statistical analysis. * indicates p<0.05

3.3 Impact of pro-longevity genes on Aß pathology and the ability of promethazine•HCl to ameliorate it

Genetic predisposition is a major risk-factor of AD. Mutations in various genes can lead to acceleration of, or protection against, the onset of AD[98, 99]. For instance, mutations in genes like PSEN1 (presenilin 1) and PSEN2 (presenilin 2) which are directly or indirectly involved in

cleaving the Aß peptide, are involved in familial AD. These mutations cause the Aß protein to get accumulated in the neurons and hence makes them dysfunctional[48]. To check the effect of various pro-longevity genes, we introduced mutants of these genes into the GCM101 and CL2355 backgrounds. crosses and generated the mutants. We then wanted to test if administering the drugs and absence of these genes might have any effect on the Aß pathology.

There are a number of genes that contribute to the longevity of *C. elegans* and two of the most important transcription factors that were found to contribute to the longevity of *glp-1* (germline less mutants) animals were *daf-16* and *nhr-49[86, 87, 100]*. Hence, we wanted to investigate if these transcription factors would have any role to play in the Aβ pathology.

3.3.1 Screening of promethazine•HCl on AGP276 (*Aβ;daf-16*) model of AD

The double mutant was obtained by crossing the Aß (muscle) expressing mutant into the double mutant of *daf-16:glp-1*. We wanted to investigate if Aß pathology is associated with the IIS/IGF (Insulin/Insulin like growth factor) pathway. Hence, we used mutants that were loss of function *daf-16*. We obtained this mutant by crossing as described in the methods section.

3.3.1.1 Effect on thrashing assay

The double mutant was found to have a deteriorating effect on the health-span compared to the GMC101 worms suggesting that the absence of *daf-16* makes it worse. Aligning with the effects of the mutant model of AD, the drug improved the health of the double mutant. The absence of *daf-16* does not make any significant difference in the thrashing, suggesting that the drug might act through this pathway or influence it (Fig. 19-A and 19-B).

Figure 20: Effect of promethazine•HCl on thrashing of AGP276 (Aβ (muscle);*daf-16*)

A. **Thrashing of GMC101 worms when treated with promethazine•HCl**

B. **Thrahsing of AGP276 (Aβ (muscle);*daf-16*) when treated with Promethazine HCl**

Experiments were performed as two independent trials with n=10 in each group. Student's unpaired t-test was used to carry out statistical analysis.

3.3.1.2 **Effect on paralysis assay**

The other important parameter was to examine the effect of paralysis on these double mutants with and without the different concentrations (100 uM and 500 uM) of the drug. The effect of paralysis was not so clear on the double mutant, although the group treated with the drug was observed to have less paralysis. The trend in the double mutant was similar to the control mutant model of AD. Also, the percent paralysis with the treated group in the mutant was comparable to the percent paralysis in the control worms. Hence, we concluded that *daf-16* could be an essential gene in bringing about the positive effect of the drug.
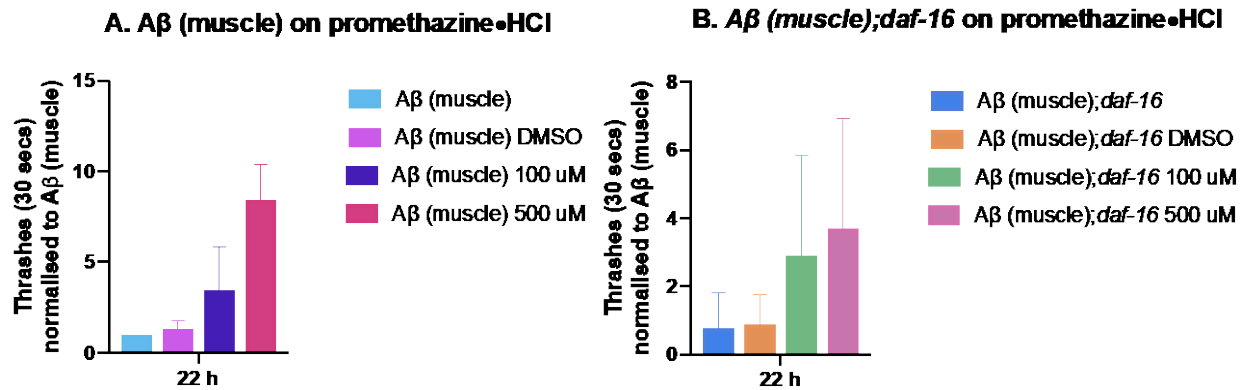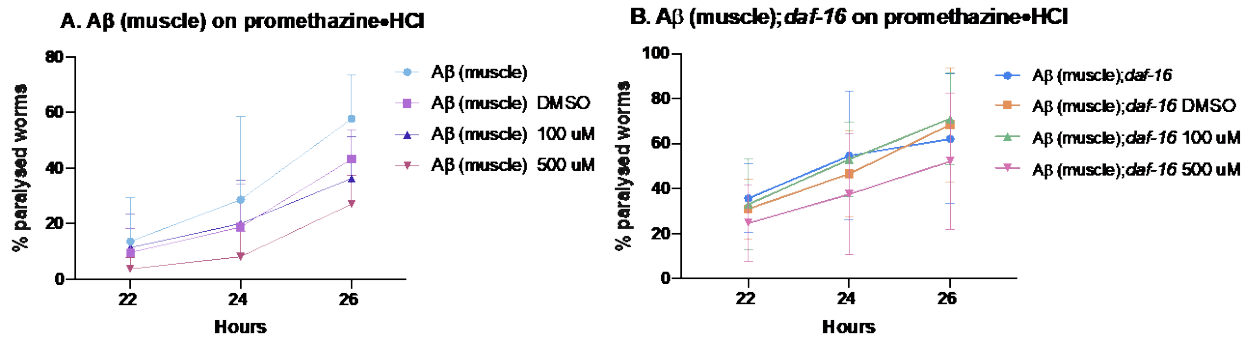
Figure 21: Effect of promethazine•HCl on paralysis of AGP276 (Aβ (muscle);*daf-16*)

A. **Thrashing GMC101 worms when treated with promethazine•HCl**

B. **Thrashing AGP276 (Aβ (muscle);*daf-16*) worms when treated with promethazine•HCl**

Experiments were performed as three independent trials with n=30 in each group. Student's unpaired t-test was used to carry out statistical analysis. * indicates p<0.05

3.3.1 Screening of promethazine•HCl on Aß(muscle);*nhr-49*

As mentioned above, we wanted to investigate the role of different genes in the Aß pathology and hence, we selected a couple of transcription factors that were involved in the lifespan extension. One of the lifespans extending genes is *nhr-49[100]*. Hence, we crossed the mutant having a LoF (loss of function) mutation into AD model of *C. elegans* and carried out the thrashing and paralysis assay.

3.3.1.1 **Effect on Thrashing assay**

The effect of the drug on these mutants was unclear. The trend was consistent with respect to the control group. The GMC101 worms that were treated with the higher concentration (500 uM) of the drug was having high number of thrashes compared to the control DMSO groups. On

the other hand, in the Aß mutant with a LoF (loss of function) *nhr*-49, there was an increase in the number of thrashes with the control DMSO group. With the treatment group, the variability seen was much higher. It may be concluded that absence of this gene might have a deteriorating effect on the pathology of the disease, and treatment with the drug has no significant effect in improving the Aß pathology.
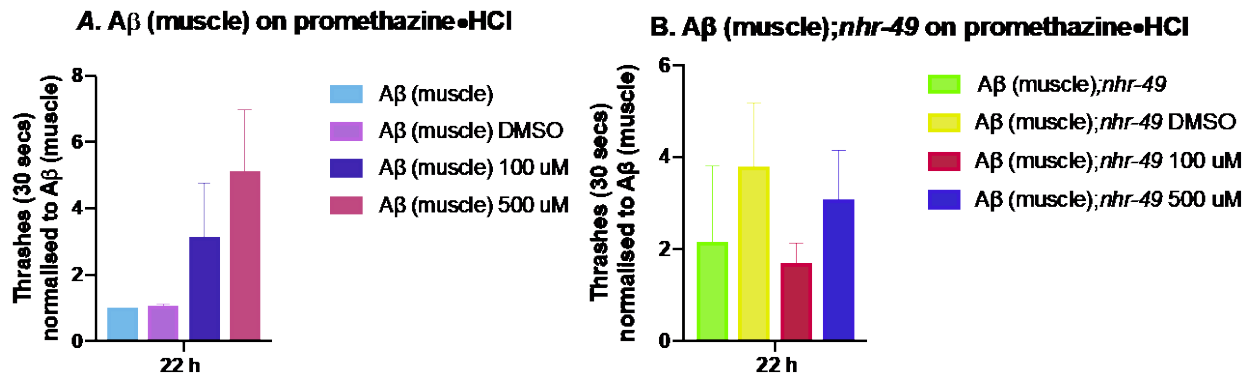
Figure 22: Effect of promethazine•HCl on thrashing of double mutant (Aβ (muscle);*nhr*-49)

A.   Thrashing of GMC101 worms when treated with promethazine•HCl

B.   Thrahsing of Aβ (muscle);*nhr*-49 worms when treated with promethazine•HCl

Experiments were performed as two independent trials with n=10 in each group. Student's unpaired t-test was used to carry out statistical analysis.

3.3.1.2 **Effect on paralysis assay**

A paralysis assay was performed on these mutants to have a clear idea of how *nhr-49* loss of function performed in an Aβ environment. Like the thrashing assay, the trend in paralysis was unclear and hence it was very difficult to conclude from the results due to high variability. Consistent with the results of thrashing assay, the DMSO control worms performed better than the lower concentration (100 uM) drug treatment group. DMSO control group in both the thrashing

and paralysis assay was found to improve Aβ pathology and hence it was concluded that DMSO

had some relationship with *nhr-49* in an Aβ environment.

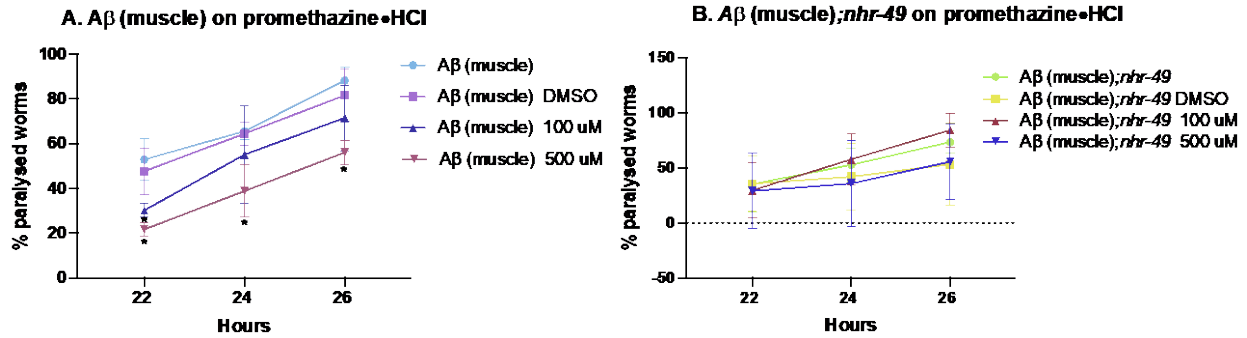**Figure 23: Effect of promethazine•HCl on paralysis of double mutant (Aβ (muscle);*nhr*-49)**

A. Effect of drug on control mutant model of AD

B. The effect of the drug on double mutant Aβ(muscle);*nhr-49*

Experiments were performed as two independent trials with n=10 in each group. Student's unpaired t-test

was used to carry out statistical analysis.

4.0 Discussion and Conclusion

Temperature stress causes a proteotoxic environment that leads to aggregation of Aβ protein in the muscle irrespective of age. We observed that exposing the animals to higher temperature or to temperature stress aggravates Aβ pathology by accumulation of Aβ protein in the muscle of one mutant (GMC101) and in the neuron of the other mutant (CL2355). Since AD is an age-related disorder, we hypothesized that age would worsen the Aβ pathology in both these mutant models (GMC101 and CL2355). GMC101 worms inherently showed reduced mobility defects compared to the WT animals. With age, these worms were found to have decreased mobility, but the effect was comparable to the WT animals. On the other hand, the CL2355 strain showed a decline in the mobility with age but had improved mobility than the WT animals.

We further investigated the effect of temperature and age together and expected the effect to be additive. However, it was observed that AD did not worsen with age in this model of *C. elegans*. This result was aligned with our previous observation of age not having any deteriorating effect on AD. This suggested the fact, that the "so called" Aβ model of *C. elegans* only expressed the protein in the developmental stages in the worms' life and hence had nothing to do with the component of age. But, temperature stress (25 °C) was enough to bring cause defects in protein homeostasis that brought about aggregation of this protein in the muscle of the animal. The aggregation of this protein mimicked the Aβ pathology. Hence, this could be used as an Aβ pathology model to screen various potential drug candidates.

Promethazine•HCl has the potential to improve Aβ pathology in the mutant model. After establishing a model that resembled the Aβ model, we tested promethazine•HCl on *C. elegans*.

Promethazine•HCl is a marketed anti-histamine that is indicated in allergic conditions and in motion sickness[101, 102]. This drug has been shown to extend lifespan of *C. elegans* and our experiments on WT lifespan confirmed the previously established results. We hence tested the drug on Aβ models of *C. elegans* and we observed that it improved Aß pathology by delaying the paralysis in the mutant expressing the protein in the muscle (GMC101) and by increasing the number of thrashes in the both the mutant expressing the protein in the muscle and the neuron (GMC101 and CL2355).

daf-16 and *nhr-49* play an important role in the Aβ pathology and *daf-16* might have a role to play in the mechanism of promethazine•HCl. We found that the Aβ;*daf-16* double mutant had a deteriorating health-span (thrashing and paralysis) when compared to the GMC101 worms. This suggested that DAF-16 is an important transcription factor for the improvement of *Aβ* model of Alzheimer's disease. When these double mutants were treated with promethazine•HCl, there was no significant change in the health-span of the worms when compared to the GMC101 worms. This suggested that the DAF-16 transcription factor is necessary for the effective action of the drug.

The Aβ;*nhr-49* double mutant showed deteriorating effects in thrashing and paralysis when compared to the Aβ model. When these mutants were treated with promethazine•HCl, the results were highly variable. It was seen that the DMSO controls had a positive effect, which was comparable to the higher concentrations (500 uM) of the drug. On the other hand, the lower concentration (100 uM) of the drug had no effect on both thrashing and paralysis. This makes the interpretation complicated and we can conclude that NHR-49 (transcription factor), promethazine•HCl and DMSO have some effect on each other which is visible in the phenotype of the animal.

Overall, the Aβ model of *C. elegans* helps us understand one small aspect of Alzheimer's disease. It takes into consideration complex effects of the disease pathology. We could also mimic the complex neurodegenerative pathology. Working with this model allows us to potentially screen thousands of drug candidates and narrow them down to a handful that can be tested in mice. Another important reason to support this model would be the *in vivo* aspect of the studies that high throughput screens don't provide.

5.0 Future directions

Once we established a model based on the phenotypic changes of the mutants, we could check the mRNA and protein levels in the animal. Tagging the amyloid-β with a GFP (green fluorescent protein) would be another way of quantifying the protein of interest. This would help us visualize the protein aggregation and quantify its expression. We could also perform sequencing of worms under various stressors and analyze the different set of genes that are upregulated or downregulated to get a clear idea of how the genetics are influenced by presence of the Aβ protein. Once we have these set of genes, we could select a few and examine the effect of alterations in these genes in an amyloid rich environment. We could also combine different mutant models of Aβ and tau to confirm the reconsiderations of the amyloid hypothesis.

Extending this model to different drug candidates would be the next step in the project, once a robust model is established. There have been high throughput screens that have been carried out on worms, but most of these are carried out on WT animals. Performing high throughput screens on our Aβ model would help us to evaluate the potential of these drug candidates against this pathology. The drug classes that can be tested are those influencing the mitochondrial homeostasis, drugs that are already established to have an influence on the studied transcription factors – DAF-16 and NHR-49, and drugs that have found to impact heat shock proteins in the worm. The rationale behind testing these drugs would be that all these have an influence on the Aβ pathology in one way or the other.

In general, although it is almost next to impossible to mimic the exact AD environment and trying to treat the disease, *C. elegans* provides us a platform to mimic the genes involved in

the complex cellular biology of Aβ pathology. It is a useful model for understanding the convoluted biology and helps us study the therapeutic effect of the various potential molecules for Aβ pathology prevention. Lastly, this model as a whole, teases out the complicated nature of Aβ pathology and allows us to test various effects rapidly. Hence, the focus of this project was mainly to develop, establish and validate a *C. elegans* model to test potential drugs that would be able to help prevent the disease.

Appendix A Abbreviations

Term	Abbreviation
AD	Alzheimer's disease
C. elegans	*Caenorhabditis elegans*
Aβ	Amyloid beta
PCR	Polymerase chain reaction
nhr-49	Nuclear hormone receptor
EOAD	Early-onset AD
LOAD	Late-onset AD
PPARα	Peroxisome proliferator-activated receptor alpha
AChE	Acetyl choline esterase
NMDA	N-methyl-D-aspartate
HTS	High throughput screening
MCI	Mild cognitive impairment
ROS	Reactive oxygen species
APP	Amyloid precursor protein
NFT	Neurofibrillary tangles
AICD	APP intracellular domain
PSEN1	Presenilin 1
PSEN2	Presenilin 2
APOE	Apolipoprotein E
DIC	Differential interference contrast

E. coli	*Escherichia coli*
L1	Larval stage 1
L2	Larval stage 2
L3	Larval stage 3
L4	Larval stage 4
RNAi	RNA interference
NGM	Nutrient growth medium
CGC	Caenorhabditis genetics center
DMSO	Dimethyl sulfoxide
$MgSO_4$	Magnesium sulfate
HCl	Hydrochloric acid
NaCl	Sodium chloride
dNTP	Deoxy nucleotide triphosphate

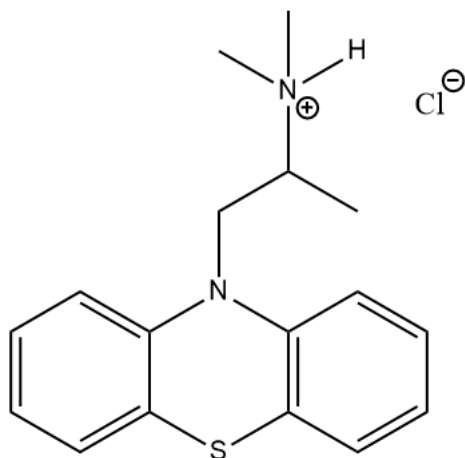# Appendix B Reagents

Figure 24: Structure of promethazine•HCl

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