

THESIS

DRUG REPURPOSING OF RAPAMYCIN MITIGATES AMYLOID BETA
ASSOCIATED PROTEOTOXICITY IN CAENORHABDITIS ELEGANS

Submitted by

Samirah Alkhudaydi

Department of Environmental and Radiological Health Sciences

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Master's Committee:

Advisor: Julie Moreno

Luke Montrose
Olivia Arnold
Frederic.Hoerndli

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ABSTRACT

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DRUG REPURPOSING OF RAPAMYCIN MITIGATES ALZHEIMER'S DISEASE

ASSOCIATED PROTEOTOXICITY IN CAENORHABDITIS ELEGANS

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Alzheimer's disease (AD) is the leading cause of dementia worldwide, affecting an estimated 55 million people and projected to exceed 130 million by 2050. Defined neuropathologically by the extracellular accumulation of amyloid-beta ($A\beta$) peptides and intracellular neurofibrillary tangles of hyperphosphorylated tau, AD currently lacks disease-modifying therapies capable of halting or reversing neurodegeneration. The proteotoxic accumulation of $A\beta$ is a central pathogenic event, and strategies that enhance endogenous clearance mechanisms represent a compelling therapeutic area.

Rapamycin (sirolimus), a macrolide allosteric inhibitor of the mechanistic target of rapamycin complex 1 (mTORC1), is one of the most robustly validated pharmacological extenders of lifespan across model organisms and has demonstrated neuroprotective potential in mammalian AD models. Its capacity to provide mTORC1-mediated suppression of autophagy and to reduce neuroinflammation positions it as an attractive drug-repurposing candidate for AD. However, the extent to which rapamycin can mitigate $A\beta$ proteotoxicity in *Caenorhabditis elegans* remains unexplored, and leveraging this model organism offers a powerful opportunity to study the mechanism by which rapamycin-mediated mTOR inhibition interacts with amyloid- β aggregation, clearance pathways, and proteostasis in the context of Alzheimer's-related neurodegeneration.

In this study, we used two well-established transgenic *Caenorhabditis elegans* models of A β proteotoxicity: the muscle-specific temperature-inducible strain CL4176 (Pmyo-3::A β ₁₋₄₂) and the pan-neuronal strain CL2355 (Psnb-1::A β ₁₋₄₂). Following an empirical dose-finding strategy across four sequential titration experiments, 20 μ M rapamycin was selected as the working concentration.

Rapamycin treatment (20 μ M) significantly delayed A β -induced paralysis in CL4176 animals, extending the time that 50% of the population was paralyzed (PT50) by approximately 1.8 hours (~23% relative to vehicle; log-rank $p = 0.0017$). Automated locomotion analysis further demonstrated that rapamycin improved peristaltic speed, center-point speed, and wavelength while markedly reducing mobility idle time ($p < 0.05$ to $p < 0.0001$). Swimming parameters as wave initiation rate, swimming speed, dynamic amplitude, and wave attenuation were similarly improved, collectively indicating a broad restoration of neuromuscular function. In the pan-neuronal CL2355 strain, rapamycin significantly rescued the A β -induced chemotaxis deficit, restoring the chemotaxis index toward control strain levels (one-way ANOVA, $p = 0.015$) without altering behavior in the non-transgenic CL2122 control. Kaplan–Meier lifespan analysis revealed that rapamycin significantly extended survival in CL2355 animals (log-rank, Bonferroni-adjusted $p < 0.0001$, with the most pronounced effect observed in the A β -expressing strain. Combined survival–locomotion analysis further demonstrated that rapamycin preserved neuromuscular healthspan, delaying the onset of age-related motor decline relative to vehicle-treated A β animals. At the molecular level, Western blot analysis using anti-A β immunoblotting revealed that rapamycin selectively reduced the accumulation of higher-molecular-weight A β oligomeric species (25 kDa and 60 kDa; $p < 0.05$ each), while monomeric and lower oligomeric A β levels were not significantly altered.

Taken together, these findings demonstrate that rapamycin confers consistent, broad-spectrum neuroprotection across orthogonal measures of A β proteotoxicity in *C. elegans*, extending from acute paralysis and locomotion to chemosensory behavior, longevity, and healthspan. The selectivity of rescue in A β -expressing animals supports a mechanism engaged with proteotoxic pathology. These results establish *C. elegans* as a tractable platform for evaluating mTOR-directed therapies and strengthen the rationale for further preclinical investigation of rapamycin as a disease-modifying strategy in Alzheimer's disease.

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CHAPTER 1: INTRODUCTION

1.1 Alzheimer's Disease: A Growing Global Health Burden

Alzheimer's disease (AD) is a chronic, age associated neurodegenerative disorder and the most prevalent cause of dementia worldwide, accounting for an estimated 60–70% of all dementia cases¹. Clinically, AD is defined by the progressive and irreversible loss of cognitive function, in particular episodic memory, language, executive processing, and visuospatial ability, ultimately culminating in a profound impairment of activities of daily living and loss of independence^{1,2}. Beyond its devastating impact on patients and their families, AD imposes an enormous socioeconomic burden on healthcare systems. In the absence of effective disease-modifying therapies and with a growing aging population, with projections exceeding 74.7 million affected adults by 2030 and more than 130 million by 2050². AD progresses through a continuum of increasingly severe stages. Before the onset of dementia, many individuals undergo a prodromal phase known as mild cognitive impairment (MCI), which is characterized by measurable decrements in memory and executive function that exceed normal age-related changes but do not yet significantly compromise everyday functioning³. A substantial proportion of individuals with amnesic MCI will advance to clinically diagnosable AD within several years^{3,4}. In the early dementia stage, patients exhibit worsening episodic recall, word-finding difficulties, impaired spatial orientation, and a reduced capacity to plan or execute complex tasks⁴. With continued disease progression, neuropsychiatric symptoms such as agitation, apathy, and depression become increasingly prominent, and patients progressively lose independence in activities of daily living⁴. Late-stage AD is characterized by severe cognitive impairment, motor deterioration, dysphagia,

incontinence, and the loss of basic physiological self-regulation, with most patients eventually succumbing to complications such as aspiration pneumonia or systemic infections ^{3,4}.

1.2 Neuropathological Hallmarks of Alzheimer's Disease

At the neuropathological level, AD is defined by characteristic molecular and structural abnormalities in specific brain regions that account for its clinical manifestations ^{1,5}. The two principal pathological hallmarks are the extracellular accumulation of amyloid-beta ($A\beta$) peptides and the intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein⁵. These lesions are accompanied by a series of secondary pathological features that contribute to neuronal dysfunction, including synaptic loss, microglial activation, reactive astrogliosis, oxidative injury, mitochondrial dysfunction, cerebrovascular pathology and progressive atrophy of the disease vulnerable brain regions, most notably in the entorhinal cortex and hippocampus ⁵⁻⁷.

1.2.1 Amyloid precursor protein processing and $A\beta$ generation

The $A\beta$ peptide is produced through sequential proteolytic cleavage of the amyloid precursor protein (APP), a type-I transmembrane protein expressed ubiquitously and particularly enriched at neuronal synapses^{7,8}. APP can be cleaved via two competing pathways (Figure 1). In the *non-amyloidogenic* pathway, APP is first cleaved by α -secretase within the $A\beta$ domain, preventing the production of intact $A\beta$ and generating large, soluble N- terminal (sAPP α) fragments that has been considered to be neuroprotective ⁹. 2.) the *amyloidogenic* pathway cleaves APP by β -site APP-

cleaving enzyme 1 (BACE1), producing a membrane fixed C-terminal fragment (C99) that is subsequently processed by the γ -secretase complex to release $A\beta$ peptides of variable length, primarily $A\beta_{1-40}$ and $A\beta_{77,99}$. The balance between these two pathways is regulated by subcellular localization of APP, membrane lipid composition and cellular stress, and the shift toward amylogenic processing is a key feature of AD pathogen^{8,98,9}.

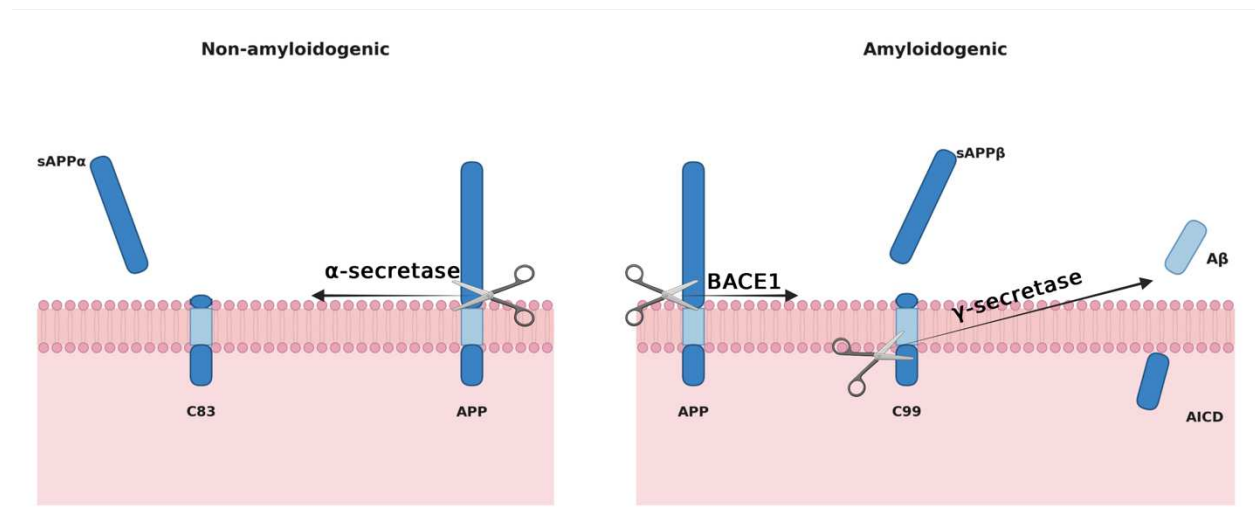


Figure 1. Proteolytic processing of amyloid precursor protein (APP). APP is a type-I transmembrane protein cleaved by two competing pathways. In the non-amyloidogenic pathway (left), α -secretase cleaves within the $A\beta$ domain to release the soluble, neuroprotective sAPP α fragment and a membrane-bound C83. In the amyloidogenic pathway (right), β -site APP-cleaving enzyme 1 (BACE1) generates sAPP β and the C99 fragment retaining the intact $A\beta$ domain; γ -secretase then liberates $A\beta$ peptides of variable length, predominantly $A\beta_{1-40}$ and $A\beta_{1-42}$. Figure created with BioRender.com

1.2.2 $A\beta$ isoforms, aggregation and oligomeric toxicity

$A\beta_{1-40}$ is the most abundant $A\beta$ species under physiological conditions, whereas $A\beta_{1-42}$ is present at lower absolute concentrations but substantially more hydrophobic and exhibit a greater tendency to adopt β -sheet conformation^{9,10}. This structural preference provides a markedly higher aggregation potential and $A\beta_{1-42}$ is consequently among the earliest and most consistently detected

pathological species in post-mortem AD brains and in cerebrospinal fluid biomarker studies^{5,7,9}.

Accumulation of A β , especially in soluble oligomeric forms, is thought to initiate a cascade of neurodegenerative events culminating in synaptic dysfunction and neuronal degeneration¹⁰.

These oligomers disrupt glutamatergic signaling, impair long-term potentiation (LTP), alter dendritic spine morphology, and initiate abnormal signaling cascades involving NMDA receptors, metabotropic glutamate receptors thereby producing synaptic dysfunction well in advance of overt plaque deposition and clinically detectable cognitive decline (Figure 2)^{9,10}.

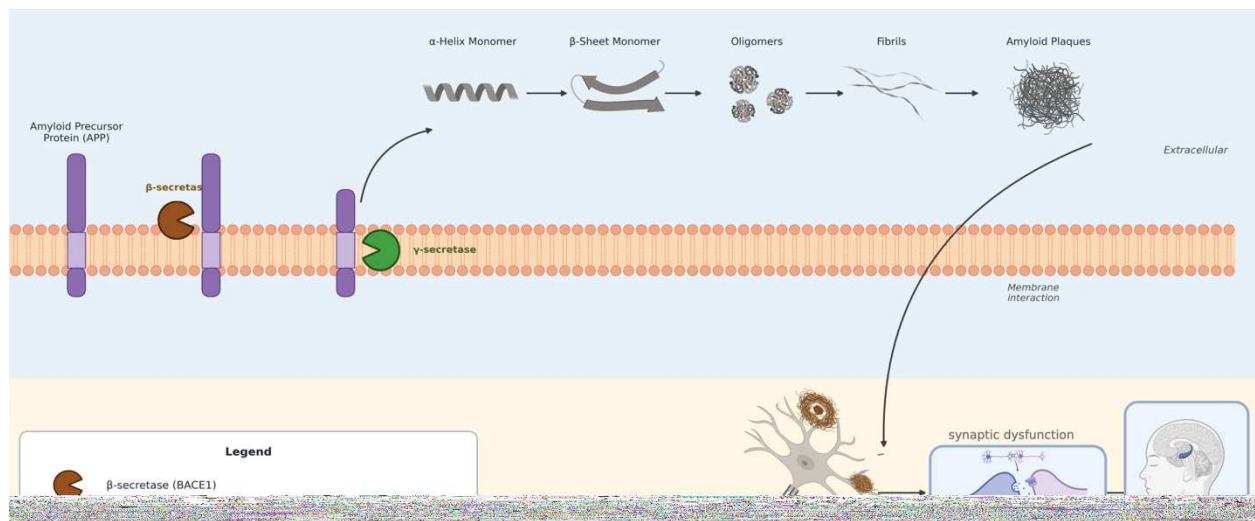


Figure 2. Schematic overview of amyloid precursor protein (APP) processing, A β aggregation, and the resulting synaptic dysfunction in Alzheimer's disease. APP is a type-I transmembrane protein that, in the amyloidogenic pathway, undergoes sequential proteolytic cleavage at the plasma membrane. β -site APP-cleaving enzyme 1 (β -secretase / BACE1) first cleaves APP on the extracellular side. The γ -secretase cleaves within the transmembrane domain, liberating A β peptides into the extracellular space. Newly released A β initially adopts an α -helical conformation but undergoes a conformational transition to a β -sheet-rich structure. β -sheet monomers progressively assemble into soluble oligomers, fibrils, which ultimately deposit as extracellular amyloid plaques. Soluble oligomeric and fibrillar A β species interact with neuronal membranes, where they disrupt synaptic signaling. The cumulative loss of synaptic integrity ultimately leads to Alzheimer's disease. Figure created with BioRender.com.

1.2.3 Tau pathology and neurofibrillary tangles

Tau is a microtubule-associated protein enriched in neuronal axons, where it stabilizes microtubules and contributes to axonal transport¹¹. In AD, tau undergoes extensive post-translational modification, most notably site-specific hyperphosphorylation, acetylation, and truncation, which progressively reduce its affinity for microtubules and promote its folding into paired helical filaments that coalesce into intracellular neurofibrillary tangles¹². The topographic distribution of NFT pathology follows a stereotyped trajectory described by Braak staging, beginning in the transentorhinal cortex and extending into the hippocampal formation, limbic system, and eventually the neocortex; critically, the spatial spread of tau pathology correlates more tightly with the pattern of neuronal loss and with the severity of cognitive impairment than does A β plaque burden¹³. Although A β and tau were historically regarded as parallel lesions, substantial experimental evidence now supports a sequential model in which A β pathology precedes and facilitates the spread of tau pathology, while tau accumulation mediates much of the downstream neuronal injury, a relationship captured in contemporary revisions of the amyloid cascade hypothesis^{7,11}.

1.2.4 The amyloid cascade hypothesis

The amyloid cascade hypothesis, first formulated in the early 1990s, proposes that the abnormal accumulation of A β is the initiating event in AD pathogenesis and that subsequent tau pathology, synaptic dysfunction, neuroinflammation, and neurodegeneration are downstream consequences of proteotoxic stress⁷. Oligomer-centric hypothesis emphasizes that soluble, low-molecular-weight A β assemblies, rather than fibrillar plaques, are the principal drivers of early synaptic failure and that persistent accumulation reflects an imbalance between A β generation and clearance^{6,7,14}. The recent, clinical achievements of anti-amyloid immunotherapies such as Lecanemab and

Donanemab provide proof-of-concept that reducing A β burden can slow cognitive decline, but their modest effect sizes and safety liabilities highlight the need for complementary approaches that act through endogenous proteostatic pathways¹⁵.

1.3 Aging: The Principal Risk Factor for Alzheimer's Disease

Aging is by far the most prevalent risk factor for late-onset AD: the incidence of the disease approximately doubles every five years after the age of 65, such that by the ninth decade of life the cumulative risk of developing AD exceeds one in three². Many of the biological processes that drive physiological aging also create a cellular environment that is permissive for the development and progression of neurodegenerative pathology¹⁶. Understanding the interface between aging biology and AD is therefore central to the rational design of disease-modifying therapies.

1.3.1 The hallmarks of aging

The hallmarks of aging are identified as a set of interconnected cellular and molecular processes that collectively contribute to age-related decline¹⁷. Among the most salient in the context of AD are the loss of proteostasis, mitochondrial dysfunction, disabled autophagy, cellular senescence, deregulated nutrient sensing and altered intercellular communication¹⁷. These hallmarks function as components of a densely interconnected network, where dysfunction in one domain can propagate to others.

1.3.2 Convergence of aging biology with AD pathogenesis

Several hallmarks of aging converge on processes that are central to AD. Loss of proteostasis reduces the capacity of neurons to fold, refold, and degrade misfolded proteins, directly enabling the accumulation of A β oligomers and pathological tau species^{10,18}. Mitochondrial dysfunction reduces neuronal energy supply and increases the production of reactive oxygen species,

contributing to synaptic failure and to further proteotoxic stress¹⁰. Disabled macroautophagy, discussed in detail below, impairs the clearance of damaged organelles and aggregated proteins^{14,19,20}. Deregulated nutrient sensing, such as sustained activation of the mechanistic target of rapamycin (mTOR) signaling pathway, suppresses autophagic flux and shifts cellular physiology toward an anabolic, pro-aging state^{21,22}. These converging pathways position aging biology, mTOR pathway and proteostatic networks, as attractive targets for intervention in AD.

1.4 Proteostasis and the Autophagy-Lysosomal Pathway

1.4.1 The cellular proteostasis network

Protein homeostasis, or proteostasis is upheld by strictly regulated systems of protein production, assembly, and degradation, with autophagy serving a crucial function¹⁴. This network comprises molecular chaperones (including the HSP70, HSP90, and small HSP families), co-chaperones, the ubiquitin–proteasome system (UPS), and the autophagy–lysosomal pathway (ALP)¹⁸. In neurons, which are post-mitotic and long-lived, proteostatic accuracy is particularly critical because damaged proteins and organelles cannot be diluted through cell division and must instead be actively cleared¹⁸. Of the degradative arms, the UPS handles soluble, short-lived, and mono-/poly-ubiquitinated substrates, whereas the ALP is uniquely capable of degrading long-lived proteins, protein aggregates that are too large for the proteasome, and entire organelles^{18,21,22}. The ALP is therefore of particular importance in the clearance of aggregation-prone species such as A β oligomers and fibrillar tau.

1.4.2 Autophagy: types and mechanism

Autophagy is a catabolic process that sequesters cytoplasmic constituents for lysosomal degradation^{18,20,23}. Three principal forms are distinguished based on the mode of cargo delivery:

macroautophagy, microautophagy, and chaperone-mediated autophagy. Macroautophagy, hereafter referred to simply as autophagy, is the most extensively characterized and the most relevant to the clearance of aggregated proteins^{20,23}. It is initiated by the nucleation of a double-membrane structure known as the phagophore, which progressively elongates and sequesters cytoplasmic cargo before closing to form a double-membraned vesicle, the autophagosome^{20,23}. The autophagosome subsequently fuses with the lysosome to generate an autolysosome, within which cargo is degraded by lysosomal hydrolases and the resulting macromolecular building blocks are recycled to support cellular biosynthesis and bioenergetics²³. Mechanistically, autophagy is organized by a chain of command of autophagy-related (ATG) proteins^{20,23}. Initiation is driven by the ULK1 complex (which integrates upstream nutrient and energy signals from mTORC1 and AMPK²⁰.

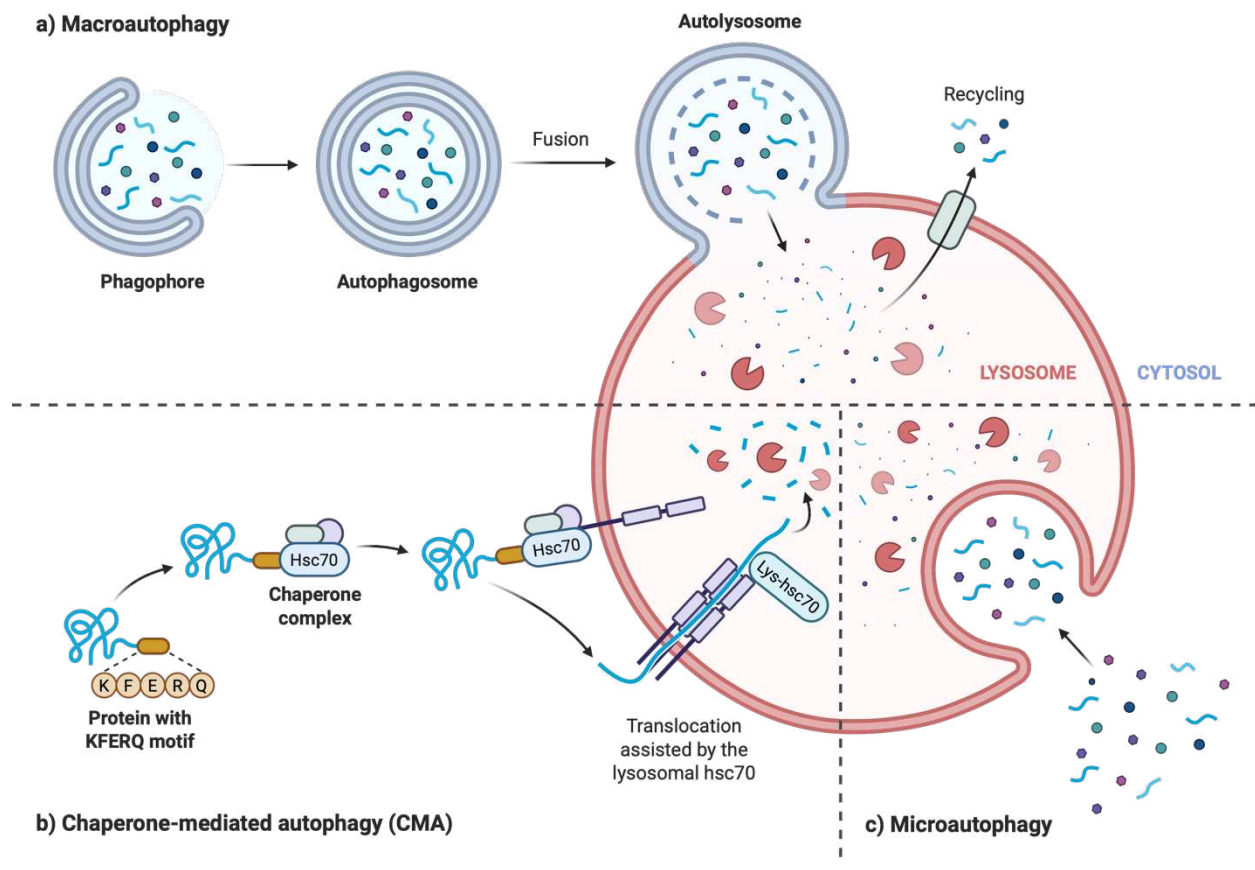


Figure 3. The three principal pathways of autophagy merge at the lysosome. Schematic representation of macroautophagy, chaperone-mediated autophagy (CMA), and microautophagy, the three mechanisms by which cytosolic cargo is delivered to the lysosome for degradation and recycling. **(a) Macroautophagy.** A double-membrane structure termed the *phagophore* nucleates in the cytosol and elongates to sequestering soluble proteins, protein aggregates, and damaged organelles. Membrane closure produces a sealed *autophagosome*, which subsequently fuses with the lysosome to form an *autolysosome*. Inside the autolysosome, lysosomal hydrolases (depicted as red enzymes) degrade the engulfed cargo degrading them to smaller substances which are exported back to the cytosol via lysosomal membrane recycling pathway. **(b) Chaperone-mediated autophagy (CMA).** Cytosolic substrate proteins bearing a KFERQ-like pentapeptide motif are selectively recognized by the cytosolic chaperone Hsc70 (and its co-chaperones), forming the substrate–chaperone complex. The complex is targeted to the lysosomal surface, where the substrate binds to its receptor and drives its multimerization into the active translocation complex. The substrate is unfolded and translocated across the lysosomal membrane in a process assisted by a luminal pool of Hsc70, after which it is rapidly degraded by lysosomal enzymes. Unlike macroautophagy, CMA delivers cargo one protein at a time and does not require vesicle formation. **(c) Microautophagy.** Cytosolic material is internalized directly through the lysosomal limiting membrane, generating intraluminal vesicles that are subsequently degraded by resident enzymes. Figure created with BioRender.com.

1.4.3 Autophagic dysfunction in Alzheimer's disease

Clinically, Autophagy has been implicated in a wide spectrum of human diseases, including cancer, neurodegenerative disorders as well as infectious diseases¹⁹. Impaired autophagic function has been associated with various protein aggregation disorders²³. Specifically, the progression of AD has been linked with dysregulation of autophagy^{17,18}. Post-mortem AD brains show an accumulation of immature autophagic vacuoles consistent with defective autophagosome–lysosome fusion and reduced lysosomal proteolytic capacity^{23,24}. This dysfunction results in defective clearance of pathogenic protein species and exacerbation of proteotoxic stress²⁴. Experimentally, pharmacological enhancement of autophagy reduces A β and tau burden, restores synaptic function, and improves behavioral outcomes in diverse AD models, whereas autophagy suppression accelerates pathology^{18,23,24}. Collectively, these observations position autophagy as both a mechanistic driver of AD and a therapeutically tractable target.

1.5 The mTOR Signaling Pathway in Health and Diseases

1.5.1 mTORC1 and mTORC2: composition and function

The mechanistic target of rapamycin (mTOR) is a conserved serine/threonine kinase that integrates signals from nutrients, growth factors to coordinate anabolic and catabolic metabolism^{24,25}. mTOR exists in two distinct multiprotein complexes, mTORC1 and mTORC2²⁵. mTORC1 consist of mTOR, the scaffolding protein Raptor, mLST8, PRAS40, and DEPTOR; it is activated at the lysosomal surface in response to amino acids, growth-factor signaling, and abundant cellular energy, and it promotes protein synthesis, lipogenesis, nucleotide synthesis, and mitochondrial

biogenesis while simultaneously suppressing autophagy^{24,25}. mTORC2, contains mTOR, Rictor, mSIN1, mLST8, and DEPTOR, regulates cell survival, cytoskeletal dynamics¹⁸. Whereas mTORC1 is acutely and potently inhibited by rapamycin, mTORC2 is largely rapamycin-insensitive over short time scales^{24,25}.

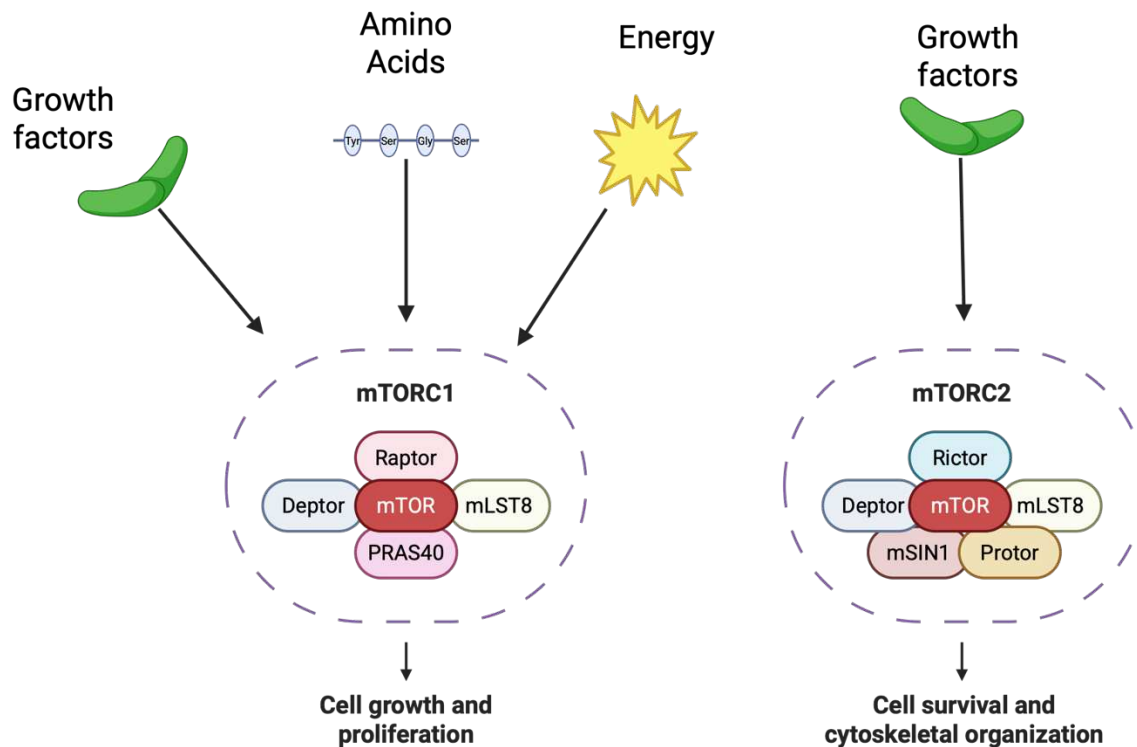


Figure 4. Composition, upstream inputs, and downstream functions of mTORC1 and mTORC2. The mechanistic target of rapamycin (mTOR) is assembled into two structurally and functionally distinct multi-protein complexes, mTORC1 and mTORC2, each integrating different upstream cues to coordinate cellular metabolism, growth, and survival. mTORC1 (left) contains protein Raptor (regulatory-associated protein of mTOR) mLST8 and the two endogenous inhibitors PRAS40 and Deptor, once active, mTORC1 promote anabolic processes suppressing catabolic processes such as autophagy, thereby driving cell growth and proliferation. mTORC2 (right) shares the catalytic mTOR subunit and the regulatory components mLST8 and Deptor with mTORC1 but is uniquely defined by the scaffold protein Rictor (rapamycin-insensitive companion of mTOR) and Protor (protein observed with Rictor). mTORC2 is activated primarily by growth-factor signaling to regulate cell survival, metabolism, and actin cytoskeletal organization. Figure created with BioRender.com.

1.5.2 mTORC1 as a master regulator of autophagy

mTORC1 acts as a key negative regulator of autophagy by preventing the initiation of autophagosomes²². When active (Figure 5A) mTORC1 phosphorylates ULK1 at Ser637 & Ser757, disrupting its interaction with AMPK and suppressing ULK1 kinase activity; it also phosphorylates ATG13 and, via S6K1, components of the class-III PI3K complex, thereby preventing autophagosome initiation^{21,22}. Pharmacological or physiological inhibition of mTORC1 for example, by caloric restriction, amino-acid withdrawal, or rapamycin rapidly releases ULK1 from inhibition, permits phagophore assembly, and promotes upregulation of the lysosomal-autophagic machinery (Figure 5B)^{22,25}.

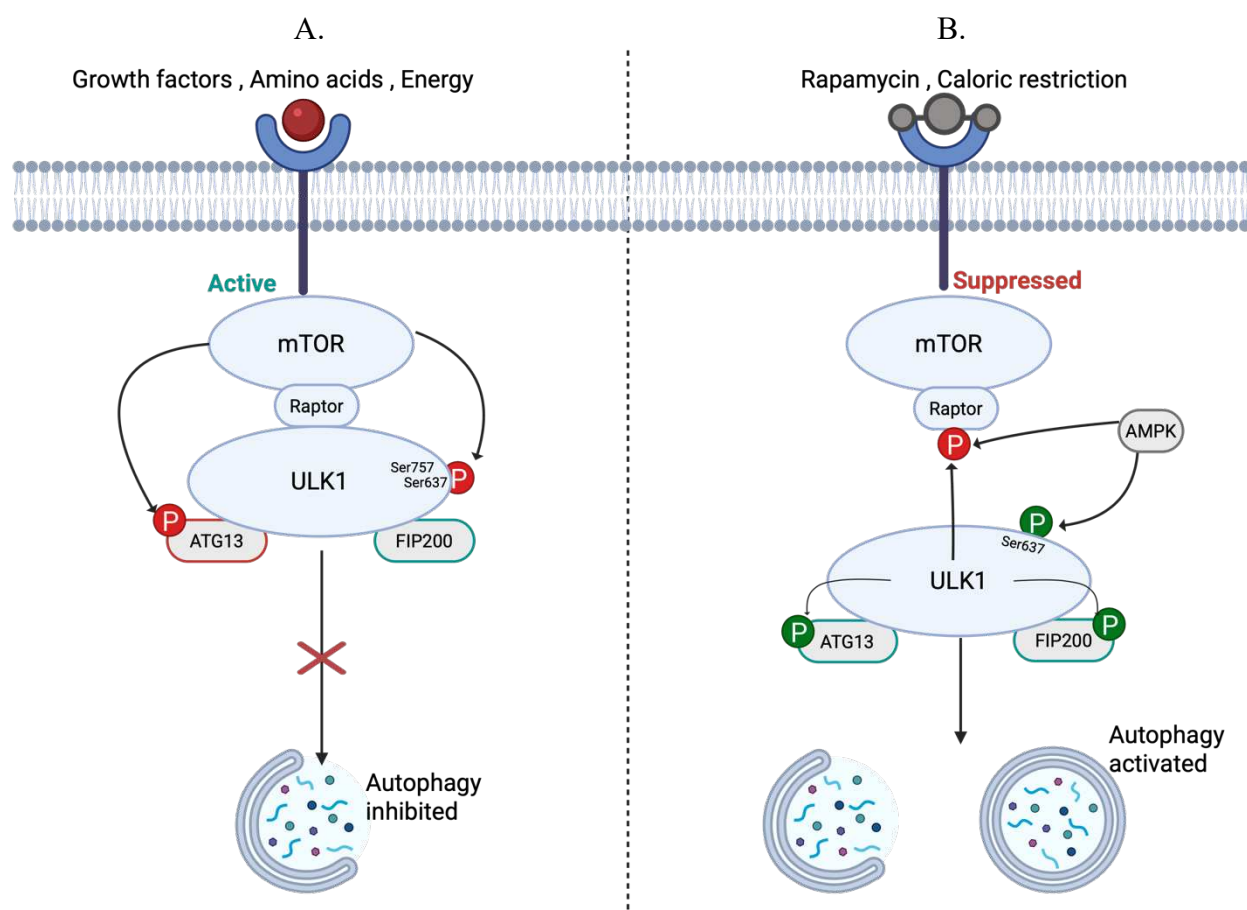


Figure 5. mTOR–ULK1 signaling controls autophagy initiation. Schematic representation of the mTOR–ULK1 under nutrient-replete (left, *Active*) and mTOR-suppressed (right, *Suppressed*) conditions.

(Left, Active state): Under nutrient/growth-factor signaling, activated mTORC1 (mTOR–Raptor complex) phosphorylates ULK1 at the inhibitory sites Ser757 and Ser637 (red P). This inhibitory phosphorylation prevents ULK1 from activating its downstream partners ATG13 and FIP200, thereby blocking initiation of autophagosome biogenesis (red X). **(Right, suppressed state):** Upon mTORC1 inhibition either pharmacologically (e.g., Rapamycin) or through energetic stress, AMPK phosphorylates Raptor (red P) to inactivate mTORC1 and directly activates ULK1 by phosphorylation at Ser637 (green P). Active ULK1 in turn phosphorylates ATG13 and FIP200 (green P), driving assembly of the ULK1 initiation complex and triggering autophagosome formation. **Color code:** red phosphate groups (P) indicate inhibitory phosphorylation events; green phosphate groups indicate activating phosphorylation events. Vesicles at the bottom represent nascent autophagosomes. *Figure created with BioRender.com.*

1.5.3 mTORC1 hyperactivation in Alzheimer's disease

Multiple independent studies report aberrantly elevated mTORC1 signaling in post-mortem human AD brain tissue and in transgenic mouse models of AD, as evidenced by increased phosphorylation of canonical mTORC1 substrates including S6K1, 4E-BP1, and ULK1 (Ser757) ^{21,25}. Crucially, mTOR hyperactivation appears to operate in a self-reinforcing loop with A β pathology: A β oligomers themselves promote mTORC1 signaling, while mTORC1 hyperactivation suppresses autophagic clearance of A β , thereby intensifying the proteotoxic burden ^{21,25}. Together, these observations identify mTORC1 as a critical molecular interface linking A β -driven proteotoxic stress, disabled autophagy, and progressive neuronal degeneration, and they provide a strong mechanistic rationale for pharmacological mTORC1 inhibition as a disease-modifying strategy.

1.6 Rapamycin: From Discovery to Therapeutic Candidate

1.6.1 Discovery and molecular mechanism

Rapamycin (sirolimus) is a macrolide compound originally isolated in the early 1970s from the bacterium *Streptomyces hygroscopicus* in a soil sample from Rapa Nui (Easter Island)²⁵. Initially developed as an antifungal and then repurposed as an immunosuppressant for the prevention of solid-organ transplant rejection, rapamycin was eventually recognized as the prototypical inhibitor

of mTORC1²⁵. Mechanistically, rapamycin binds with high affinity to the 12-kDa intracellular receptor FKBP12, and the resulting FKBP12–rapamycin complex docks onto the FRB domain of mTOR, allosterically inhibiting the catalytic activity of mTORC1 toward canonical substrates such as S6K1 and 4E-BP1²⁵. Selective inhibition of mTORC1 signaling by rapamycin, while preserving mTORC2 activity over short durations, removes the inhibitory constraint on autophagy and enhances lysosomal-autophagic function^{22,25}.

1.6.1.1 Rapamycin Pharmacokinetic

Rapamycin have low and variable oral bioavailability (approximately 14%), demonstrating extensive first-pass metabolism by intestinal and hepatic cytochrome P450 3A4 and active efflux by P-glycoprotein^{26,27}. The compound is highly lipophilic, distributes widely into tissue with a large apparent volume of distribution, and is eliminated slowly, with a terminal half-life of approximately 60–80 hours that necessitates a loading dose to achieve steady-state concentrations rapidly^{26,27}. *C. elegans* lacks a true one-to-one ortholog of human CYP3A, the nematode genome encodes between 76–82 genes that independently expanded cytochrome P450 into 16 families²⁸. Based on sequence similarity and gene architecture, these genes fall into loose associations with the mammalian CYP2, CYP3, and CYP4 clades, but they don't correspond to true one-to-one orthologs²⁹. The single gene most often cited as CYP3A4's closest relative is CYP-13A12 which shares only about 32% amino-acid identity with CYP3A4 and functions in xenobiotic clearance^{30,31}. No *C. elegans* CYP has been shown to catalyze the demethylation reactions that CYP3A4/3A5 perform on rapamycin and the reported data for rapamycin in *C. elegans* is drug delivery rather than metabolism³².

1.6.1.2 Rapamycin Pharmacodynamics

Rapamycin does not act by directly neutralizing a disease-causing substrate; rather, it forms a tertiary complex with the intracellular receptor FKBP12. This complex allosterically inhibits mTORC1 kinase activity, initiating a signaling cascade whose downstream consequences (detailed below) are the ultimate determinants of the observed pharmacological effect²⁵.

1.6.1.3 Direct versus indirect drug effect of Rapamycin

Pharmacological effects are broadly classified as direct or indirect. A direct effect occurs when a drug's action tracks its plasma or tissue concentration in real time, typically through reversible occupancy of a receptor or enzyme active site, so that the response rises and falls in parallel with drug exposure³³. An indirect effect, in contrast, arises when a drug modulates the production, degradation, or activity of a downstream physiological mediator rather than acting instantaneously on the measured response itself; the resulting effect is delayed relative to drug exposure and may persist after the drug has been cleared, a behavior classically described by indirect-response pharmacodynamic models³³. Rapamycin demonstrates an indirect-acting drug: its binding to FKBP12 and inhibition of mTORC1 kinase activity is rapid, but the downstream consequences relevant to A β proteotoxicity, namely release of ULK1/ATG13 inhibition, induction of autophagosome formation, and lysosomal clearance of aggregated protein, unfold over a substantially longer time-course²².

1.6.1.4 Dose, concentration, and dosage.

These three terms are frequently used interchangeably but describe distinct pharmacological concepts.

- **Dose** is the absolute amount of drug administered to an organism at one time, typically expressed in mass or mass per unit body weight (e.g., mg or mg/kg)³⁴.

- **Concentration** is the amount of drug present per unit volume of a defined compartment, whether the external medium surrounding the organism (e.g., μM in culture medium) or an internal compartment such as plasma or tissue (e.g., ng/mL). critically, the nominal concentration applied externally is not the same quantity as the concentration ultimately achieved inside the organism, since the latter depends on absorption, distribution, metabolism, and excretion³⁴.
- **Dosage**, or dosage regimen, refers to the schedule or timing on how a given dose is administered over time, that is, the amount per administration, the frequency of administration, and the total duration of treatment³⁴.

1.6.2 Rapamycin as a geroprotector

Rapamycin is one of the most robust lifespan-extending pharmacological agents identified to date, extending lifespan across multiple model organisms, including yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, and mice³⁵⁻³⁷. Notably, rapamycin extends murine lifespan even when administered late in life, suggesting that a portion of its pro-longevity effects is mediated through mechanisms that remain accessible in aged tissues including the rescue of autophagic flux and the attenuation of chronic, age-associated inflammation³⁸. These conserved, late-life benefits have generated substantial interest in mTORC1 inhibition as a generalized strategy for intervening in age-related disease, and they provide a particularly compelling rationale for testing rapamycin in disorders of protein aggregation and aging, foremost among which is AD.

1.6.3 Preclinical evidence in Alzheimer's disease model

In transgenic rodent models of AD, rapamycin has been reported to reduce A β plaque burden, diminish soluble A β oligomer levels, attenuate tau hyperphosphorylation, preserve synaptic integrity, and improve performance on hippocampus-dependent learning and memory tasks^{18,21,25}.

These benefits are consistently accompanied by markers of enhanced autophagic flux, decreased neuroinflammation, and improved cerebral perfusion^{18,25}. Importantly, several studies report that rapamycin retains therapeutic efficacy when administered after the onset of overt pathology, a finding with direct translational relevance given that AD is typically diagnosed after substantial neuropathological change has already occurred^{25,38}.

1.7 *Caenorhabditis elegans* as a Model for Alzheimer's Disease Research

Despite the mechanistic insights provided by transgenic rodent models, progress in AD drug discovery is constrained by the high cost, long duration, and comparatively low throughput of mammalian studies. The nematode *Caenorhabditis elegans* offers a powerful model that overcomes many of these limitations³⁹. *C. elegans* has a short and reproducible lifespan (approximately 2–3 weeks under standard conditions), a completely mapped cell lineage (959 somatic cells in the adult hermaphrodite), a fully sequenced genome with extensive functional annotation, and a defined nervous system of 302 neurons that form the synaptic connectome³⁹. It is flexible to genetic manipulation (including by RNA interference via simple bacterial feeding) and can be cultured on solid or liquid media at low cost, enabling high-throughput pharmacological and genetic screening with statistical power that is impractical in mammalian systems³⁹.

1.7.1 ADME in C. elegans

C. elegans exhibits a distinct pharmacokinetic profile that must be considered when interpreting drug treatment studies in this organism. Compounds administered via the bacterial food source or seeded directly onto culture plates must first cross the collagenous cuticle and the pharyngeal and intestinal epithelia, barriers that substantially restrict and slow the uptake of many xenobiotics^{40,41}. Once absorbed, internal distribution and clearance are mediated in part by the excretory system,

most notably the H-shaped excretory canal cell, a single large cell that extends the length of the animal and functions analogously to the vertebrate renal tubule, collecting fluid and metabolic waste for expulsion through the excretory duct and pore⁴¹. *C. elegans* additionally expresses a broad repertoire of cytochrome P450 enzymes, UDP-glucuronosyltransferases, and ATP-binding cassette (ABC) transporters that carry out phase I/II biotransformation and active efflux of xenobiotics, providing a conserved, if incompletely characterized, absorption-distribution-metabolism-excretion (ADME) system^{40,41}.

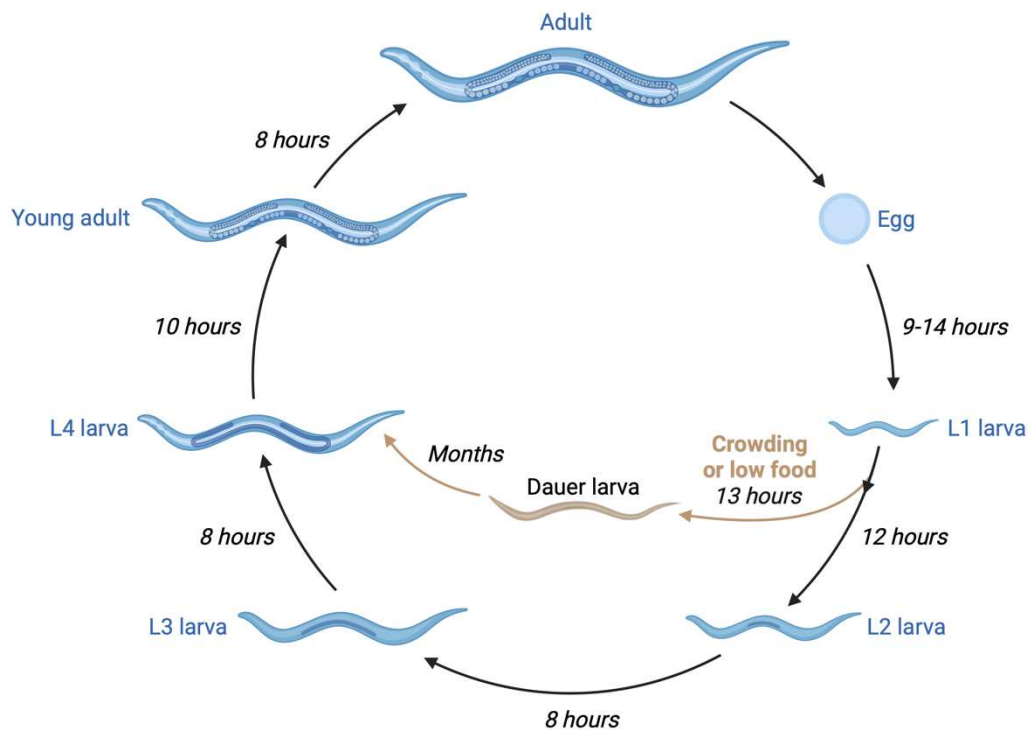


Figure 6. The life cycle of *Caenorhabditis elegans*. Schematic of the developmental stages of *C. elegans* under standard laboratory conditions. Following fertilization, embryos hatch as L1 larvae (9–14 h post egg-laying) and progress through four larval stages: L1, L2, L3, and L4, before maturing into young adults and reproductively active adults. Approximate stage durations are indicated next to each arrow. Adult

hermaphrodites produce fertilized eggs, completing the cycle. Under unfavorable environmental conditions as overcrowding or limited food availability, the L1 larvae enter an alternative developmental route and form dauer larvae, a stress-resistant, non-feeding diapause stage. Dauer larvae can persist for several months once conditions improve; they re-enter the reproductive cycle into L4 larvae and resuming normal development. *Figure created with BioRender.com.*

1.7.1 Transgenic A β -expressing strains: CL4176 and CL2355

C. elegans does not naturally produce human A β ; transgenic strains have been engineered to express human A β in specific tissues, enabling the experimental investigation of A β proteotoxicity in a whole organism³⁹. Two strains are of specific applicability to the present work. The CL4176 strain expresses human A β_{1-42} selectively in body-wall muscle under the control of a temperature-inducible *myo-3* promoter system; transgene expression is suppressed at the permissive temperature (typically 16 °C) and induced upon upshift to the restrictive temperature (typically 23–25 °C), producing a paralysis phenotype that is quantitative, reproducible, and highly sensitive to pharmacological modulation of A β proteotoxicity⁴². The CL2355 strain, on the other hand, expresses human A β_{1-42} pan-neuronally under the *snb-1* promoter, producing behavioral deficits that demonstrate the cognitive and behavioral phenotypes more directly analogous to those observed in AD⁴³. Together, these strains allow for a parallel examination of muscle-specific (proteotoxic, peripheral) and neuronal (behavioral, central) consequences of A β expression in a single experimental platform.

1.8 Gap in Knowledge and Study Rationale

Although rapamycin has demonstrated neuroprotective effects in several mammalian models of neurodegeneration, its impact on tissue-specific A β expression systems remains unaddressed^{5,21,25}. Specifically, the extent to which rapamycin can rescue A β -associated phenotypes in the muscle-specific paralysis model (CL4176) and the pan-neuronal behavioral model (CL2355) of *C. elegans*

has not been characterized. Addressing this gap is important for two reasons. First, tissue-specific A β expression systems isolate the contribution of A β proteotoxicity from confounding effects of upstream APP processing, thereby providing a clean readout of clearance-oriented interventions. Second, behavioral phenotypes in the CL2355 strain provide a functionally meaningful readout that maps more directly onto cognitive and behavioral outcomes in human AD. Clarifying the therapeutic potential of rapamycin in these *C. elegans* systems will therefore contribute to the further validation of *C. elegans* as a scalable platform anti-AD for drug discovery.

1.9 Hypothesis and Specific Aims

Based on the above-mentioned considerations, this study proposes the following central hypothesis: rapamycin attenuates A β toxicity and improves neuronal health in transgenic *C. elegans* models of Alzheimer's disease. To test this hypothesis, the study pursues two specific aims.

Aim 1 seeks to determine whether rapamycin alleviate the toxic phenotypes in transgenic *C. elegans* expressing human A β ₁₋₄₂ in the muscle-specific CL4176 strain. The effect of rapamycin on A β -induced paralysis will first be characterized, and the window within which rapamycin delays the onset and progression of paralysis following temperature upshift. Subsequently, the effect of rapamycin on A β -induced locomotion deficits will be evaluated in the same strain, using locomotion performance as a functional readout of muscle coordination and A β -associated neuromuscular dysfunction. To confirm these functional readouts at the molecular level, Western blot analysis will be performed to quantify monomeric and oligomeric A β species and assess the impact of rapamycin on proteotoxic protein accumulation in this strain.

Aim 2 focuses on determine whether rapamycin confers neuroprotection in transgenic *C. elegans* expressing human A β ₁₋₄₂ in the neuron-specific CL2355 strain. The effect of rapamycin on behavioral phenotypes will be assessed by quantifying the Chemotaxis Index in rapamycin-treated and vehicle-treated animals, to determine whether rapamycin attenuates neuronal A β -mediated neurosensory deficits. In addition, lifespan assays will be performed to determine whether the longevity effects of rapamycin are preserved, enhanced, or attenuated in the presence of A β -driven proteotoxicity. Molecular confirmation will be provided through Western blot analysis to evaluate the effect of rapamycin on A β accumulation in the neuronal-expressing strain.

CHAPTER 2: MATERIALS AND METHODS

2.1 Preparation of Rapamycin

Rapamycin (sirolimus; molecular weight 914.17 g/mol) was purchased from Cayman Chemical Company (Ann Arbor, MI, USA; item no.13346) at $\geq 98\%$ purity. A 10 mM stock solution was prepared by dissolving the lyophilized powder in dimethyl sulfoxide (DMSO; Sigma–Aldrich), aliquoted into single-use volumes to avoid repeated freeze–thaw cycles and stored at $-20\text{ }^{\circ}\text{C}$ protected from light. Working solutions were prepared by diluting the stock in DMSO and added to molten nematode growth medium (NGM, cooled to $\sim 37\text{ }^{\circ}\text{C}$) immediately before pouring, such that the final DMSO concentration in all plates (treated and vehicle control) did not exceed 0.2% (v/v). This DMSO concentration has previously been shown to have no detectable effect on *C. elegans* lifespan, locomotion, or A β -induced phenotypes (Wang et al., 2014; Calahorro & Ruiz-Rubio, 2011). The choice of Rapamycin as a TOR-pathway inhibitor was based on its well-established ability to extend lifespan and improve proteostasis in *C. elegans* and other model organisms (Robida-Stubbs et al., 2012; Bjedov et al., 2010; Harrison et al., 2009).

2.2 Materials

Benzaldehyde ($\geq 99\%$) were purchased from Sigma–Aldrich (St. Louis, MO, USA; catalogue no.100-52-7). Petri dishes 100mm x 15 mm were obtained from Fisher scientific (catalogue no. FB0875712). The 6E10 anti-A β monoclonal antibody was purchased from BioLegend (San Diego, CA, USA; catalogue no. 803014), the anti- β -actin antibody from ABclonal (Woburn, MA, USA; catalog no.AC004) and the beta amyloid D54D2 rabbit monoclonal antibody was purchased from

Cell Signaling (catalog no. 8243). Horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody was obtained from Bio-Rad (catalog no.1706516). The protease inhibitor cocktail used for worm lysis was purchased from (Roche cOmplete™ EDTA-free, catalogue no. 11873580001).

2.2.1 Worm strains and maintenance

Four transgenic *Caenorhabditis elegans* strains were used in this study: CL4176 (dvIs27 [*Pmyo-3::A β ₁₋₄₂::let-851 3' UTR + Prol-6(su1006)*]), which expresses human A β ₁₋₄₂ in body-wall muscle in a temperature-inducible manner ^{44,45}; CL2355 (dvIs50 [*Psnb-1::A β ₁₋₄₂::3' UTR(long) + Pmtl-2::GFP*]), which drives pan-neuronal A β ₁₋₄₂ expression following a temperature upshift ⁴⁶; and the genetically matched non-A β control strains CL802 (dvIs15 [*Pmyo-3::GFP::let-851 + Prol-6(su1006)*]) and CL2122 (dvIs15 [*Pmtl-2::GFP + Punc-54::3' UTR(long)*]) (Wu et al., 2006). All strains were obtained from the *Caenorhabditis* Genetics Center (CGC; University of Minnesota, Minneapolis, MN, USA).

Worms were cultured on solid NGM plates (3 g/L NaCl, 17 g/L agar, 2.5 g/L peptone, 1 mM CaCl₂, 1 mM MgSO₄, 25 mM KH₂PO₄ pH 6.0, 5 µg/mL cholesterol) seeded with a 100 µL lawn of *Escherichia coli* OP50 as a food source, prepared as described by Brenner (1974)³⁹ and Stiernagle (2006)⁴⁷. Stock plates of CL4176, CL2355 and their controls were maintained at 16 °C. Transgene expression was induced experimentally by upshifting the temperature to 25 °C as detailed in the assay-specific sections below.

2.2.2 General Maintenance Buffers

- **M9 buffer:** 22 mM KH₂PO₄, 42 mM Na₂HPO₄, 86 mM NaCl, 1 mM MgSO₄ (added after autoclaving from a sterile 1 M stock); used for worm washes and recovery ⁴⁷.

- **S-medium:** S-basal (5.85 g/L NaCl, 1 g/L K₂HPO₄, 6 g/L KH₂PO₄, 5 µg/mL cholesterol) supplemented with 10 mL/L 1 M potassium citrate (pH 6.0), 10 mL/L trace metals solution, 3 mL/L 1 M CaCl₂, and 3 mL/L 1 M MgSO₄ ⁴⁷.

2.2.3 Synchronization

2.2.3.1 Buffers

Bleaching (alkaline hypochlorite) solution: 0.5 M NaOH, 0.8% (v/v) sodium hypochlorite, prepared fresh from concentrated stocks immediately before use ⁴⁸.

2.2.3.2 Methode

Age-synchronized populations were obtained using the alkaline hypochlorite (“bleaching”) method, which exploits the resistance of the eggshell to brief hypochlorite exposure ^{47,48}. Gravid adult hermaphrodites were collected from gravid plates by washing with M9 buffer and pelleted by low-speed centrifugation. The supernatant was discarded, and the pellet was incubated in freshly prepared bleaching solution (0.5 M NaOH, 0.8% sodium hypochlorite) for 5–7 min at room temperature with agitation, until microscopic inspection confirmed that essentially all adult bodies had dissolved while eggs remained intact. The released eggs were pelleted by centrifugation at $1,800 \times g$ for 2 min, the supernatant aspirated, and the pellet washed three times with sterile M9 buffer followed by a final wash in S-medium. The clean egg suspension was then transferred onto unseeded NGM plates and allowed to hatch overnight at 16 °C. Because hatched L1 larvae arrest development in the absence of food, this procedure yields a population that is tightly synchronized at the L1 stage upon feeding. Synchronized L1 larvae were transferred to fresh NGM plates seeded with OP50 and incubated at 16 °C until they reached the developmental stage required for each assay.

2.3 Rapamycin dose determination

To identify a working concentration that produced a biological response without overt toxicity, a preliminary dose–response experiment was performed using the temperature-inducible A β -induced paralysis phenotype of CL4176 (Section 2.4) as the read-out. Synchronized L1 larvae were exposed to NGM containing Rapamycin at 1, 10, 20, 30, 50, and 100 μ M (final DMSO matched the drug concentration), and the percentage of paralyzed worms was scored at 21 h after the temperature upshift for 2-hour interval until all vehicle group animals are dead. The lowest concentration that significantly delayed paralysis relative to the vehicle was selected as the working concentration. The results of the dose-response curves are further discussed in (Section 3.1)

2.4 Amyloid β -induced paralysis assay

The temperature-inducible paralysis assay was performed in the CL4176 strain following the procedures of Dostal & Link (2010) ⁴⁵, with minor modifications. Age-synchronized L1 larvae were transferred onto NGM plates containing either vehicle (0.2% DMSO) or 20 μ M Rapamycin (in 0.2% DMSO), seeded with OP50 as the food source. Plates were incubated at 16 °C for 48 h to allow worms to develop to the L3 stage. Muscle-specific A β _{1–42} expression was then induced by upshifting the cultivation temperature to 25 °C, and this time point was designated 0 h post-induction. Beginning at 20 h post-induction, paralysis was scored every 2 h until all the vehicle-treated worms were paralyzed (Figure 7). A worm was scored as paralyzed if it failed to propagate a complete sinusoidal body wave or moved only its head/nose region in response to gentle prodding with a platinum wire pick ⁴⁵. Each experimental condition included at least 50 worms per plate, and 1 independent biological replicate were performed. Data were expressed as

the percentage of non-paralyzed worms over time and analyzed by log-rank (Mantel–Cox) test in R then graphed on GraphPad Prism (Section 2.10).

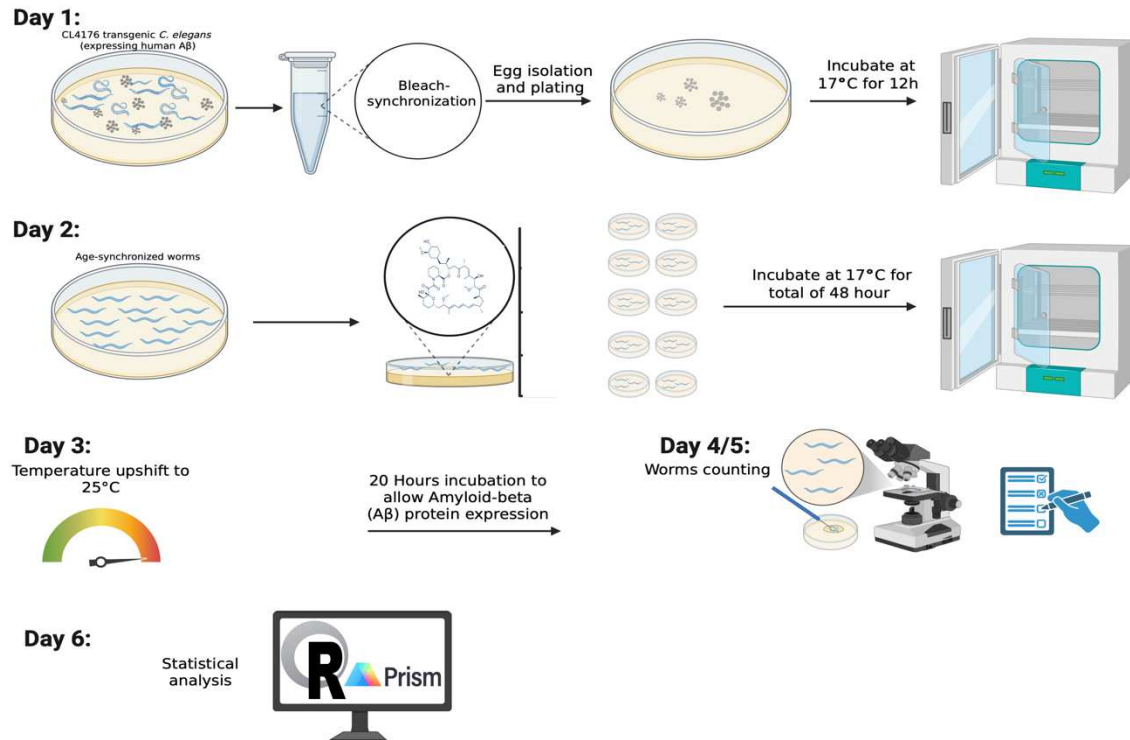


Figure 7. Experimental workflow for the temperature-inducible paralysis assay in CL4176 transgenic *C. elegans*. Schematic overview of the six-day procedure used to assess the effect of Rapamycin on muscle-specific A β _{1–42}–induced paralysis. **Day 1:** Gravid CL4176 adults (expressing human A β _{1–42} under a muscle-specific, temperature-inducible promoter) were bleach-synchronized to release eggs, which were plated onto NGM plates and incubated at 16 °C for ~12–14 h to obtain age-synchronized L1 larvae (without food source). **Day 2:** Synchronized L1 larvae were transferred onto NGM plates containing either vehicle (0.2 % DMSO) or 20 μ M Rapamycin in 0.2 % DMSO, seeded with *E. coli* OP50 as a food source, and maintained at 16 °C for a total of 48 h to allow development to the L4 stage. **Day 3:** Cultivation temperature was upshifted from 16 °C to 25 °C to trigger muscle-specific A β _{1–42} expression; this time point was designated as 0 h post-induction. Worms were then incubated at 25 °C for 20 h to permit A β protein accumulation. **Days 4–5:** Beginning at 20 h post-induction, paralysis was scored every 2 h until all vehicle-treated animals were paralyzed. Each condition included $\geq$ 50 worms per plate, with one independent biological replicate. **Day 6:** The percentage of non-paralyzed worms over time was analyzed by log-rank (Mantel–Cox) survival test in R and visualized as Kaplan–Meier curves in GraphPad Prism. Figure created with BioRender.com.

2.5 Western blotting

2.5.1: Buffers

- RIPA lysis buffer: 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1% (v/v) NP-40 (Igepal CA-630), 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM EDTA, supplemented with 1× protease inhibitor cocktail.
- TBS-Tween (TBS-T): 100 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween-20.
- 4× SDS sample buffer: 250 mM Tris–HCl pH 6.8, 8% (w/v) SDS, 40% (v/v) glycerol, 16% (v/v) β-mercaptoethanol, 0.002% (w/v) bromophenol blue (Laemmli, 1970).
- SDS-PAGE running buffer: 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS.
- Western transfer buffer: 25 mM Tris, 192 mM glycine, 20% (v/v) methanol.
- Tris-Tricine running buffer: 100 mM Tris, 100 mM Tricine, 0.1% SDS, pH 8.3 following dilution to 1x with water.
- Tricine sample buffer: 100 mM Tris-HCl, pH 6.8, 40% glycerol, 2% SDS, 0.04% Coomassie Blue G-250.
- Stripping buffer: 200 mM glycine, 0.1% SDS, 1% Tween-20, pH 2.2.

2.5.2 Method:

- Aβ Oligomers:

To quantify Aβ_{1–42} protein levels, Western blotting was carried out following the principles of Towbin et al. (1979) ⁴⁹ and the worm-specific protocol described by Wu et al. (2006) ⁴⁶. Age-synchronized CL4176 worms were cultured at 16 °C for 36 h on NGM plates supplemented with vehicle or 20 μM Rapamycin, then upshifted to 25 °C for 36 h to induce Aβ_{1–42} expression. Worms were collected from plates by washing with ice-cold ddH₂O, pelleted by centrifugation at 1,800 ×

g for 30 s, and washed three additional times to remove residual bacteria. Worm pellets were resuspended in ice-cold 1× RIPA buffer containing protease inhibitor cocktail and lysed by three cycles of freeze–thawing in liquid nitrogen and a 37 °C water bath, followed by three freeze-sonication cycles. Lysates were clarified by centrifugation at $14,000 \times g$ for 15 min at 4 °C, and total protein concentration in the supernatant was determined using the bicinchoninic acid (BCA) assay.

Equal aliquots containing 15 µg of total protein were combined with 4× SDS sample buffer, denatured at 95 °C for 5 min, briefly cooled on ice and centrifuged. Proteins were resolved on a 20% Tris–glycine SDS-PAGE gel at 120 V for ~90 min. Resolved proteins were transferred onto a 0.45 µm polyvinylidene difluoride (PVDF) membrane (Millipore, IPVH00010) at 80 V for 1 h at 4 °C in transfer buffer. Membranes were blocked with 5% (w/v) non-fat dry milk in TBS-T for 1 h at room temperature, then incubated overnight at 4 °C with rabbit monoclonal anti-Aβ D54D2 (Cell Signalling-; 1:500 in blocking buffer). After three 10 min washes in TBS-T, membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit IgG (1:1,000), washed three further times in TBS-T, and developed using an enhanced chemiluminescent (ECL) substrate. Chemiluminescent signals were captured digitally using analytik jena uvp chemstudio imager. Band intensities were quantified densitometrically in ImageJ/Fiji and Aβ signals were normalized to β-actin. At least 3 independent biological replicates were performed for each condition.

- Aβ Monomer:

To quantify Aβ_{1–42} monomer levels, Western blotting was carried out following the same technique for the oligomers with minor changes. Equal aliquots containing 15 µg of total protein were combined with Tricine sample buffer, denatured at 70 °C for 10 min, briefly cooled on ice and

centrifuged. Proteins were resolved on a 16.5% Tris–tricine SDS-PAGE gel at 100 V for ~90 min. Resolved proteins were transferred onto a 0.22 μ m nitrocellulose membrane from Azure biosystems (Catalog no. AC2107AZ) at 100 V for 30 minutes at 4 °C in transfer buffer. To enhance detection of A β , membranes were boiled in PBS for 3 min after transfer, as previously shown to expose buried epitopes recognized by 6E10 (Ida et al., 1996). Membranes then were blocked with 5% (w/v) non-fat dry milk in TBS-T for 1 h at room temperature, then incubated overnight at 4 °C with mouse monoclonal anti-A β 6E10 (BioLegend; 1:500 in blocking buffer). After three 10 min washes in TBS-T, membranes were incubated for 1 h at room temperature with HRP-conjugated goat anti-mouse IgG (1:5,000), washed three further times in TBS-T, and developed using an enhanced chemiluminescent (ECL) substrate. Chemiluminescent signals were captured digitally using Analytik Jena UVP ChemStudio imaging system. Band intensities were quantified densitometrically in ImageJ/Fiji and A β signals were normalized to β -actin. At least 1 independent biological replicate were performed for each condition.

- β -actin

Membranes were stripped using a stripping buffer, followed by three washes with TBS-T (Tris-buffered saline with 0.1% Tween-20). Stripped membranes were then blocked with 5% (w/v) non-fat dry milk in TBS-T for 1 h at room temperature. Following blocking, membranes were incubated overnight at 4 °C with mouse monoclonal anti- β -actin antibody (1:10,000 dilution). Membranes were then washed three times with TBS-T and incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (1:5,000 dilution) for 1 h at room temperature. After three additional washes with TBS-T, protein bands were visualized using enhanced chemiluminescence (ECL) substrate. Chemiluminescent signals were captured digitally using an Analytik Jena UVP ChemStudio imaging system. Band intensities were quantified by densitometric analysis using

ImageJ/Fiji software (National Institutes of Health). This stripping and reproving procedure was performed for all Western blots to confirm equal protein loading.

2.6 Chemotaxis assay

2.6.1 Materials

- CTX (chemotaxis) buffer: 5 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ (pH 6.0), 1 mM CaCl_2 , 1 mM MgSO_4 ^{50,51}.
- Chemotaxis assay plates: 25 mM phosphate buffer (pH 6.0), 1 mM MgSO_4 , 1 mM CaCl_2 , and 1.9% (w/v) agarose.

2.6.2 Method

Chemotaxis behavior was assessed in the pan-neuronal $\text{A}\beta$ strain CL2355 and its control CL2122, following the protocols of (Bargmann et al. 1993) and (Margie et al. 2013), with modifications introduced for the temperature-inducible neuronal- $\text{A}\beta$ strain. L1 age synchronized animals were cultured with vehicle (0.2% DMSO) or 20 μM Rapamycin at 16 °C for 36 h, then upshifted to 25 °C for an additional 36 h to induce neuronal $\text{A}\beta_{1-42}$ expression. Worms were harvested in CTX buffer, washed three times with the same buffer to remove bacteria, and gently pelleted with gravity without centrifuging between washes.

Chemotaxis was performed on 100 mm assay plates containing poured a day before the assay. The underside of each plate was marked with two test (T) and two control (C) spots arranged on diametrically opposite quadrants approximately 2 cm from the plate edge (Figure 8). Immediately before placing worms, 1 μL of attractant (0.1% benzaldehyde in 100% ethanol) plus 1 μL of 0.25 M sodium azide (used as an anesthetic to immobilize worms upon arrival) ⁵¹ were spotted at each test point, and 1 μL of 100% ethanol plus 1 μL of 0.25 M sodium azide were spotted at each control point. Approximately 50 worms were transferred to the center of the plate using a small drop of CTX buffer; excess buffer was carefully removed with a Kimwipe to allow free movement. Plates

were incubated for 1 h at room temperature, after which the number of worms within a defined radius around each spot was counted under a Zeiss microscope (Figure 9). The chemotaxis index (CI) was calculated as:

$$CI = (N_{\text{attractant}} - N_{\text{control}}) / N_{\text{total}}$$

where $N_{\text{attractant}}$ is the number of worms at the two attractant spots, N_{control} is the number of worms at the two control spots, and N_{total} is the total number of scored worms⁵¹. Worms that did not make it to the designated test or control area were excluded from the calculation. CI values range from -1 (perfect repulsion) to +1 (perfect attraction); values close to 0 indicate impaired chemosensory behavior. Three independent biological replicates were performed, with approximately 50 worms per group per replicate.

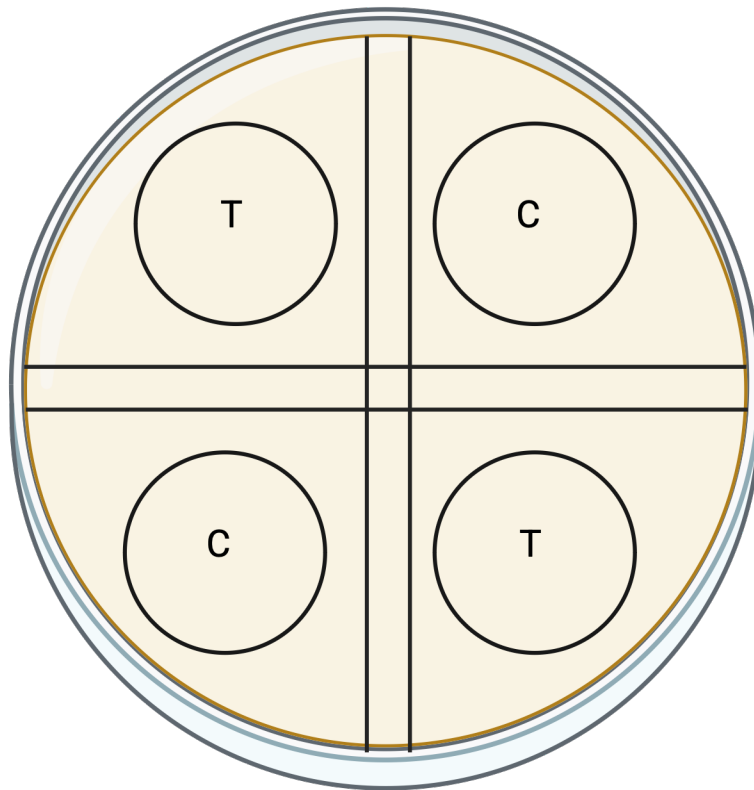


Figure 8. Template of Chemotaxis assay plate. A standard 9 cm agarose plate was marked from the underside and divided into four equal quadrants. Test (T) and Control (C) compound spots were positioned

in a counter-balanced diagonal arrangement, with the two T spots placed in opposite quadrants and the two C spots placed in the remaining diagonal, each spot located 2.5 cm from the central intersection of the four quadrants. Approximately 50 worms were pipetted at the central intersection between the dividing lines and allowed to distribute freely across the plate for the duration of the assay. At the end of the assay, only worms that had entered one of the four scoring circles were counted toward that group; worms remaining on the centerlines or outside the scoring circles were excluded. *Figure created with BioRender.com.*

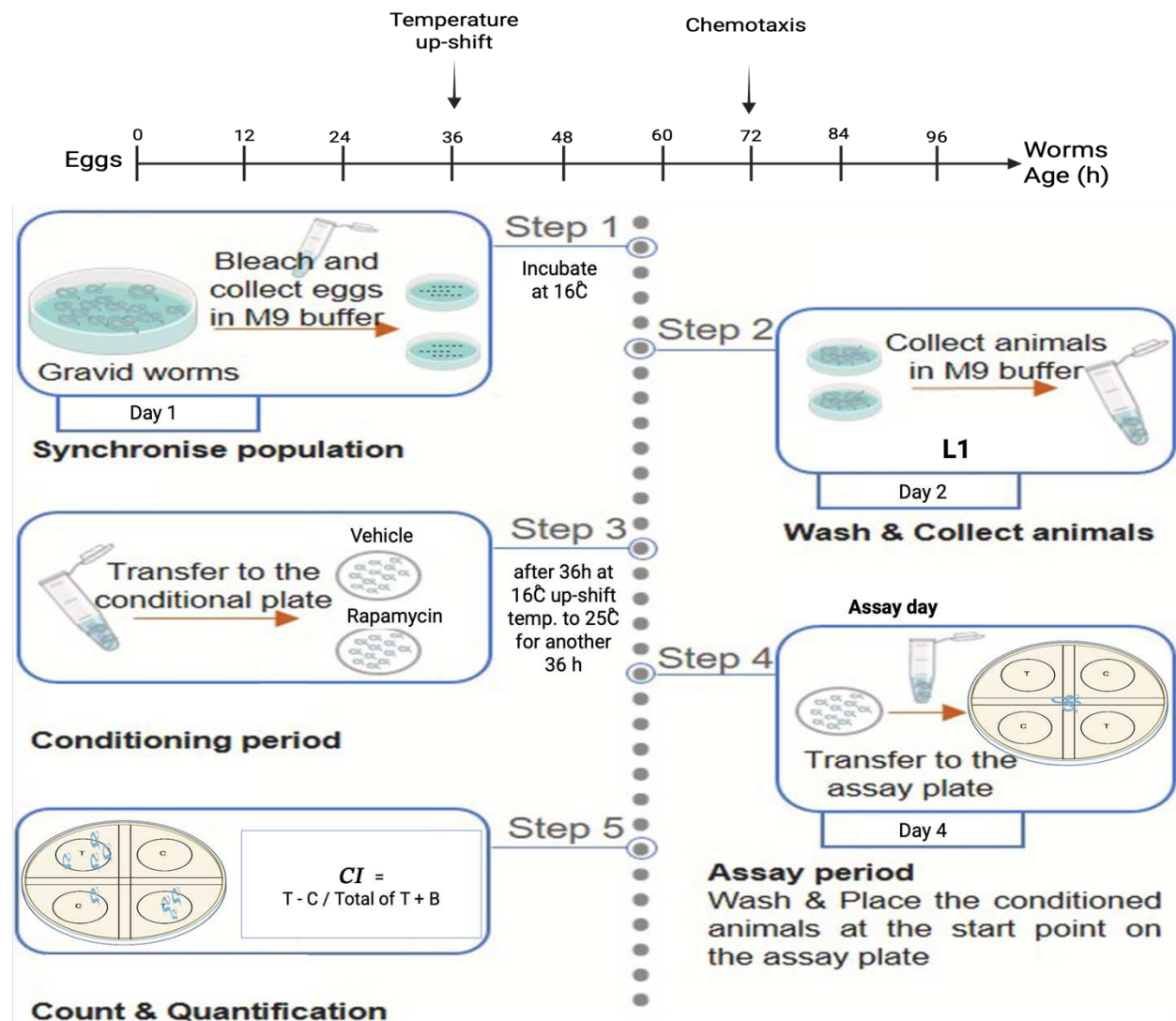


Figure 9. Experimental workflow for the chemotaxis assay in CL2355 transgenic *C. elegans*. Schematic timeline of the multi-step procedure used to assess the effect of Rapamycin on chemotactic behavior. **Step 1 (Day 1, Synchronize population):** Gravid CL2355 were treated with alkaline hypochlorite (bleach) to release eggs, which were collected in M9 buffer, plated onto NGM and incubated at 16 °C to obtain an age-synchronized population. **Step 2 (Day 2, Wash & collect):** Synchronized animals were washed from the plates with M9 buffer and pelleted by gravity. **Step 3 (Day 2, Conditioning period):** Collected animals were transferred onto NGM conditioning plates containing either vehicle (0.2 % DMSO) or 20 µM Rapamycin. Plates were maintained at 16 °C for 36 h, then up-shifted to 25 °C for an additional

36 h to induce neuron-specific A β _{1–42} expression while conditioning continued. **Step 4 (Day 4, Assay Day):** Conditioned animals were washed off the conditioning plates, briefly rinsed in CTX buffer, and pipetted onto the central intersection of a four-quadrant chemotaxis assay plate, with Test (T) and Control (C) spots arranged in a counter-balanced diagonal layout. **Step 5 (Count & Quantification):** Worms were allowed to distribute across the plate for 1 hour, after which the number of animals within each scoring circle was counted under a stereomicroscope. The chemotaxis index (CI) was calculated. *Figure created with BioRender.com.*

2.7 Lifespan Assay

Lifespan analysis was carried out using CL2355 and the matched control strain CL2122, following standard *C. elegans* lifespan procedures (Amrit et al., 2014)⁵² adapted to the temperature-sensitive A β strain.

Synchronized L4 larvae (Approximately 100 worms per group, distributed across 6 wells of a 24-well plate at ~15-20 worms per well) were transferred to each well containing 50 μ g/mL 5-fluoro-2'-deoxyuridine (FUDR) to suppress progeny production (Mitchell et al., 1979)⁵³, in the presence or absence of 20 μ M Rapamycin, and fed with concentrated OP50. Transgene expression was induced by upshifting the temperature from 16 °C to 25 °C; the 24h post the upshift was defined as day 1 of adulthood and used as the starting point for survival scoring.

Worms were scored every 48 h until the death of the last animal. Death was defined as failure to respond to gentle stimulation with a platinum pick, accompanied by the absence of pharyngeal pumping (Sutphin & Kaeberlein, 2009)⁵⁴. Animals that displayed internal hatching, vulval rupture, or visible bacterial/fungal contamination were censored at the time of last observation and excluded from survival analysis. Survival data were analyzed by the Kaplan–Meier method, and pairwise comparisons between groups were performed using the log-rank (Mantel–Cox) test on R and graphed in GraphPad Prism (Section 2.10). At least two independent biological replicates were performed.

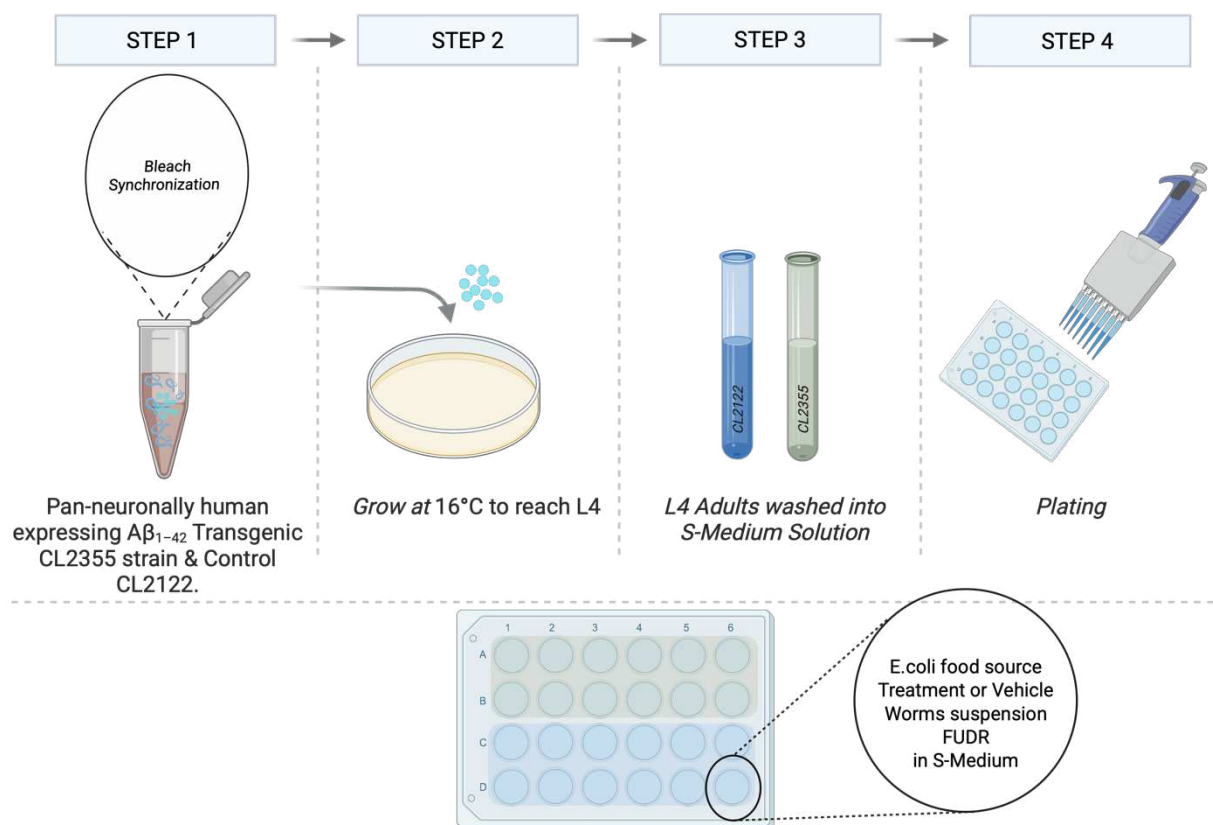


Figure 10. Experimental setup for the lifespan assay in CL2355 and CL2122 transgenic *C. elegans*. Schematic representation of the synchronization and plating workflow used to assess the effect of Rapamycin on $A\beta$ -induced lifespan changes. **Step 1 - Bleach synchronization:** Gravid adults of the pan-neuronal human $A\beta_{1-42}$ transgenic strain CL2355 and its matched control strain CL2122 were treated with alkaline hypochlorite (bleach) solution to release eggs and dissolve all post-embryonic stages, yielding an age-synchronized embryonic population. **Step 2 - L4 development:** Collected eggs were transferred onto NGM plates seeded with *E. coli* OP50 and incubated at 16 °C allowing development to the L4 stage. **Step 3 - Wash & collection:** Synchronized L4 animals from each strain were washed off NGM plates and resuspended separately in S-medium, yielding two strain-specific worm suspensions (CL2122 and CL2355). **Step 4 - Plating:** Worm suspensions were distributed into a 24-well with approximately 15–20 worms per well across six wells per condition (~100 worms per group total). **lower panel:** Plate layout of the 24-well assay. Rows A–B contained CL2355, and rows C–D contained CL2122 (match to your actual layout). Each well was filled with concentrated *E. coli* OP50, 5-fluoro-2'-deoxyuridine (FUDR), and either 0.2 % DMSO (vehicle) or 20 μ M Rapamycin in 0.2 % DMSO. The magnified circle illustrates the contents of a single well. Following plating, the cultivation temperature was up-shifted from 16 °C to 25 °C to induce $A\beta_{1-42}$ expression, and survival was scored every 48 h beginning 24 h post-upshift (Day 1 of adulthood) using a Kaplan–Meier with log-rank (Mantel–Cox) pairwise comparisons. *Figure created with BioRender.com.*

2.8 Thioflavin S fluorescence staining

The effect of Rapamycin on A β aggregation was examined by Thioflavin S (ThS) staining, a fluorescent dye that selectively binds the cross- β sheet structure of amyloid fibrils, the procedure was adapted from (Wu et al. 2006)⁴⁶ and (Link et al. 1995)⁵⁵. Age-synchronized CL2355 worms were cultured on NGM containing vehicle or 20 μ M Rapamycin at 16 °C for 36 h, then shifted to 25 °C for 36 h to induce neuronal A β_{1-42} expression.

Worms were collected in M9 buffer, washed three times to remove bacteria, and transferred onto poly-L-lysine-coated glass slides. The slides were then freeze-cracked by immersion in liquid nitrogen for ~5 min and the coverslips removed with a razor blade to permeabilize the cuticle (Duerr, 2006)⁵⁶. Exposed worms were fixed briefly in cold 100% methanol and stained with 0.125% (w/v) ThS dissolved in 50% (v/v) ethanol for 15 min in the dark at room temperature. Excess dye was removed by a 3 min destaining wash in 80% ethanol followed by two brief rinses in M9. Stained samples were mounted in ProLong Diamond (Thermofisher catalog no. P36965) under a coverslip. ThS fluorescence was visualized using a fluorescence microscope equipped with a standard GFP filter set (excitation $\approx$ 470 nm, emission $\approx$ 525 nm) at 20 $\times$ magnification. Images were acquired using identical exposure settings across treatment groups, and ThS-positive deposits in the head region were quantified in ImageJ/Fiji. At least 10 worms per condition were imaged across three independent biological replicates.

2.9 Locomotion assay

Crawling locomotion of CL4176 worms on solid NGM was assessed using automated video tracking with the WormLab imaging system and software (MBF Bioscience), following an approach similar to (Restif et al. 2014)⁵⁷ and (Ramot et al. 2008)⁵⁸. Age-synchronized CL4176

worms were cultured at 16 °C for 48 h on NGM containing vehicle or 20 μ M Rapamycin, then upshifted to 25 °C for 27 h to induce muscle-specific A β ₁₋₄₂ expression (first time point post temperature-shift to show paralysis phenotype). Worms were washed three times in M9 buffer to remove bacteria, and approximately 50 worms per condition were transferred onto an unseeded NGM plate; excess liquid was carefully blotted away with a Kimwipe to prevent worms from swimming. Locomotion was recorded for 1 min using a Zeiss microscope.

Recorded videos were imported into WormLab software for automated worm detection and tracking. Tracks shorter than 10 s, tracks that overlapped with other worms, and tracks corresponding to worms that crawled off the field of view were excluded from analysis. Crawling parameters relevant to the aims of the present study were extracted as follows:

- Peristaltic speed (μ m/s): the velocity at which the dorsoventral muscle contraction wave propagates from head to tail along the body axis; an integrated measure of forward progression that combines bending frequency and wavelength ⁵⁹.
- Centroid (centre-point) speed (μ m/s): the instantaneous velocity at which the worm's centroid (centre of mass) moves through space, averaged over the recording period ⁵⁹.
- Mobility idle time (s): the cumulative duration over the observation period during which the worm's centroid displacement falls below the WormLab "idle" threshold; this parameter reports on the propensity of worms to pause or stop crawling ⁵⁹.
- Wavelength (μ m): the distance between two consecutive peaks of the body bend during locomotion, computed from the worm's skeletonized midline ⁵⁹. 1 independent biological replicates were performed for each condition, statistical analysis (Section 2.10).

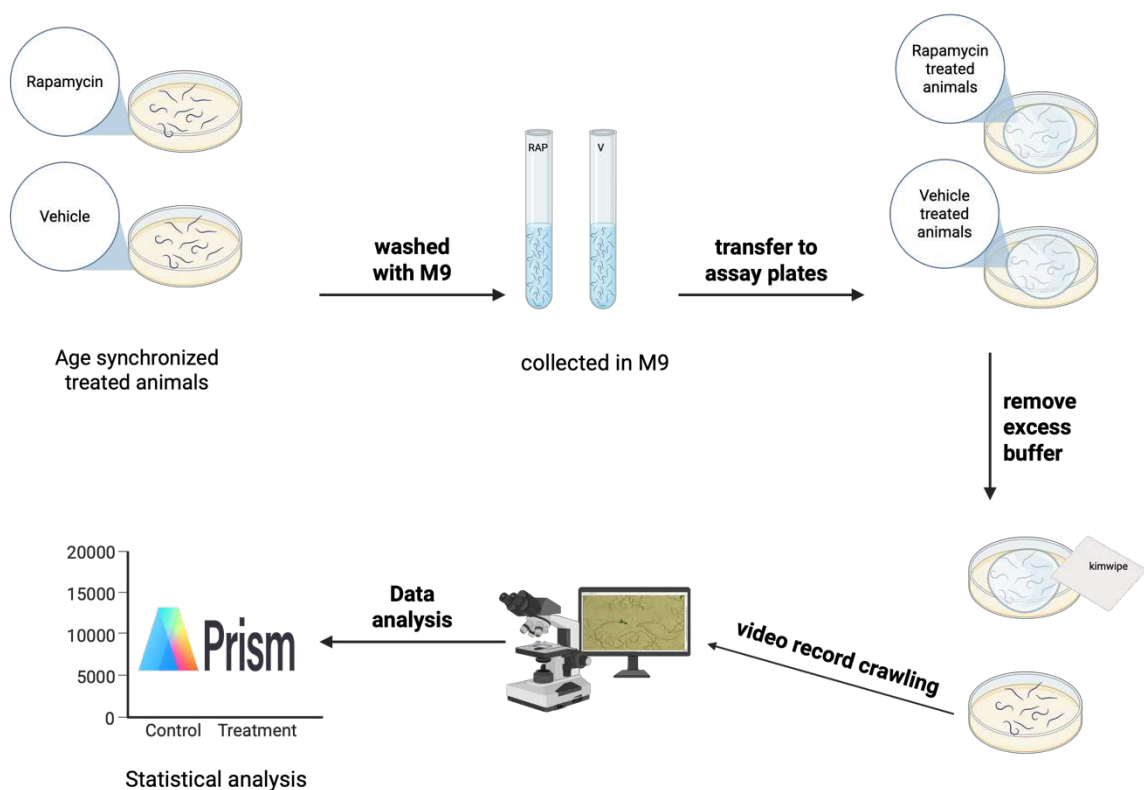


Figure 11. Experimental workflow for automated assessment of crawling locomotion in CL4176 *C. elegans*. Schematic of the procedure used to quantify the effect of Rapamycin on crawling behavior following $A\beta_{1-42}$ induction. **Step 1 - Conditioning:** Age-synchronized CL4176 transgenic worms were cultured on NGM plates containing either Vehicle (0.2 % DMSO) or 20 μ M Rapamycin at 16 °C for 36 h, followed by a temperature up-shift to 25 °C for 36 h to induce muscle-specific $A\beta_{1-42}$ expression. **Step 2 - Washing:** Treated worms from each condition were collected into separate tubes (RAP = Rapamycin; V = Vehicle) and washed three times in M9 buffer to remove residual bacteria. **Step 3 - Transfer to assay plates:** Approximately 50 worms per condition were pipetted onto unseeded NGM plates. **Step 4 - Drying:** Excess liquid was gently absorbed from the plate using a Kimwipe to prevent worms from swimming and to ensure that crawling was the behavior recorded. **Step 5 - Video recording:** Worm locomotion was recorded for 1 minute under a Zeiss microscope. **Step 6 - Tracking and analysis:** Recorded videos were imported into WormLab software (MBF Bioscience) for automated detection and tracking of individual worms. Quantified locomotion parameters (e.g., Center Point Speed) were exported and statistically compared between Vehicle- and Rapamycin-treated groups using GraphPad Prism. Figure created with BioRender.com.

2.10 Survival linked with locomotion analysis

To investigate whether Rapamycin treatment alters healthspan in addition to lifespan, a combined survival-locomotion assay was performed. This approach is based on the observation that age-

associated decline in motility precedes death in *C. elegans* and provides a sensitive index of physiological aging that can be uncoupled from chronological lifespan (Herndon et al., 2002; Glenn et al., 2004; Hahm et al., 2015; Bansal et al., 2015). The assay was performed in parallel with, and using the same experimental setup as, the lifespan assay described in Section 2.7, in the transgenic strain CL2355 and its non-transgenic control CL2122.

Briefly, age-synchronized L4 larvae (100 worms per group) were transferred to 24-well containing 50 μ g/mL FUDR in the presence or absence of 20 μ M Rapamycin, with OP50 supplied as a food source. Transgene expression was induced by upshifting the temperature from 16 °C to 25 °C, and the 24h after upshift was designated as day 1 of adulthood. Worms were scored as alive or dead every other day until the last animal had died, using the same survival criteria as in Section 2.7: animals that failed to respond to gentle stimulation with light or a platinum pick and showed no pharyngeal pumping were scored as dead, whereas animals that exploded, displayed internal hatching, or became contaminated were censored.

On each scoring day, the surviving worms in each well were video recorded for 30 seconds using a Zeiss microscope. The resulting videos were imported into WormLab software for automated detection and tracking, and two readouts measures of motor activity were extracted as a function of adult age:

- Wave initiation rate (Hz): the frequency at which new dorsoventral body waves are generated at the head and propagated along the body axis, providing a direct read-out of motor neuron and body-wall muscle output ^{57,60}.
- Overall activity: a composite WormLab index that integrates centroid displacement, posture change, and bending events into a single normalized score reflecting the global locomotor activity of each animal ⁵⁹.

By aligning per-day locomotion measurements with the corresponding survival data, the trajectory of motor decline could be examined relative to chronological age and to the time of death, allowing healthspan effects of Rapamycin to be distinguished from changes in lifespan alone. One independent biological replicates were performed. Survival data were analyzed as in Section 2.7, and locomotion parameters were compared between groups at matched time points using the statistical procedures described in Section 2.11.

2.11. Statistical analysis

All quantitative data are presented as the mean $\pm$ standard error of the mean (SEM) of at least 3 independent biological replicates, unless otherwise indicated. Statistical analyses were performed using GraphPad Prism v11.0.1 (GraphPad Software, San Diego, CA, USA) and R (version 4.4.0+526; R Core Team, 2026). For multiple-group comparisons, one-way analysis of variance (ANOVA) test was applied. Non-parametric data were analyzed using the Kruskal–Wallis test. Paralysis and lifespan data were analyzed by the Kaplan–Meier method, and survival differences between groups were compared using the log-rank (Mantel–Cox) test. A p value of < 0.05 was considered statistically significant; significance levels are indicated in figures as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

CHAPTER 3: RESULTS

3.1 Dose-Optimization strategy identifies 20 μ M Rapamycin as the Working Concentration

3.1.1 Rationale for dose-finding strategy

Although rapamycin has been used widely in *C. elegans* longevity and proteostasis studies, the published literature reports a wide range of doses, between 1 and 100 μ M and the optimal dose is highly dependent on the genetic background and purpose of the study⁵⁰⁻⁵². Before any therapeutic claims could be made about rapamycin, it was necessary to empirically identify a concentration that (i) produced a measurable, reproducible delay in A β -induced paralysis, (ii) outperformed the dimethyl-sulfoxide (DMSO) vehicle by a significant margin, and (iii) did not exhibit overt toxicity. To achieve this, a cycle of dose range study was performed in four sequential rounds: an initial broad-range of 1–100 μ M (1, 10, 50, and 100 μ M), a secondary scan of the board-range scan but match with its vehicle, a low-dose vehicle-control verification (matched DMSO), and a focused narrow-range comparison around the candidate working dose. The findings from each round are presented below.

3.1.2 Broad-range dose study

In the first round of the dose-optimization study, age-synchronized CL4176 larvae, expressing Amyloid beta in the muscle that would undergo paralysis with a temperature change, were transferred to NGM plates supplemented with vehicle (1% DMSO) or rapamycin at one of four concentrations: 1, 10, 50, or 100 μ M. Following the standard 48- hour (h) conditioning at 16°C and temperature upshift to 25°C, the proportion of non-paralyzed animals was scored at 2-h intervals from 21 to 37 hrs post-upshift (Figure 10). Pairwise survival comparisons across the five

conditions were performed using the Log-rank test with Bonferroni correction for multiple testing; the resulting p-values are summarized in Table 1.

Three observations emerged from this initial study. First, none of the four tested rapamycin concentrations produced the marked rightward shift in the paralysis curve; all five curves remained tightly clustered between 27 and 37 hrs post-upshift. Second, the two lowest concentrations (1 and 10 μM) produced curves that were statistically significant from vehicle ($p = 1.1 \times 10^{-9}$ and $p = 6.4 \times 10^{-4}$, respectively), but the paralysis trajectory of these low-dose treatment was slightly worse than the vehicle control. Third, the two higher concentrations (50 and 100 μM) were non-significant (n.s.) compared to the vehicle and were significantly different from the lower doses (e.g., 1 μM vs 50 μM , $p = 4.0 \times 10^{-15}$).

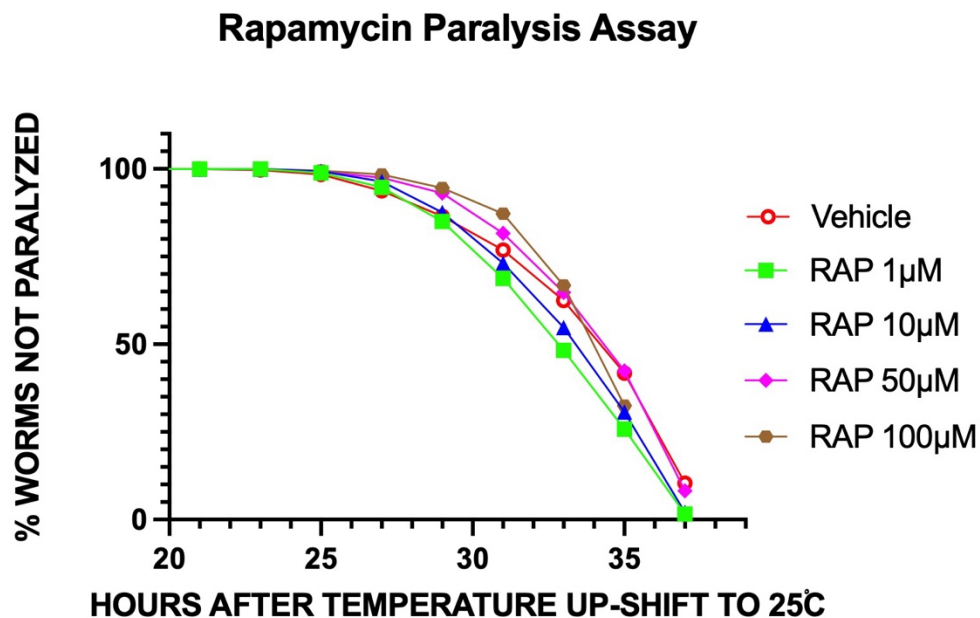


Figure 12 Broad-range dose-response of Rapamycin on $\text{A}\beta$ -induced paralysis in CL4176 transgenic *C. elegans*. Age synchronized CL4176 worms were cultured at 16 $^{\circ}\text{C}$ on NGM containing 0.2 % DMSO (Vehicle) or Rapamycin (RAP) across a four-log dose range (1, 10, 50, and 100 μM) muscle-specific $\text{A}\beta_1$ –

42 expression was induced by up-shifting the temperature to 25 °C (defined as 0 h on the X-axis). Beginning at 20 h post-induction, paralysis was scored every 2 h until all vehicle-treated animals were paralyzed. Data are plotted as the percentage of non-paralyzed worms over time; ≥ 50 worms per condition, $n = 1$ independent biological replicate(s). Pairwise comparisons between each Rapamycin concentration and Vehicle were performed by log-rank test in R and curves were generated in GraphPad Prism.

Table 1. Pairwise Log-rank comparisons (Bonferroni-adjusted) for the broad-range dose-response shown in Figure12. Paralysis data from CL4176 worms treated with rapamycin (RAP) at 1, 10, 50, or 100 μM (relative to a single 0.2% DMSO vehicle). Statistical comparisons were performed by log-rank (Mantel–Cox) test in R with Bonferroni correction for multiple comparisons. Significant differences ($p < 0.05$) from vehicle are indicated; n.s., not significant =1.

Pairwise comparison	Bonferroni-adjusted p-value
Vehicle vs RAP 1 μM	1.1×10^{-9}
Vehicle vs RAP 10 μM	6.4×10^{-4}
Vehicle vs RAP 50 μM	n.s.
Vehicle vs RAP 100 μM	n.s.
RAP 1 μM vs RAP 50 μM	4.0×10^{-15}
RAP 1 μM vs RAP 100 μM	3.7×10^{-9}
RAP 10 μM vs RAP 50 μM	2.6×10^{-7}
RAP 10 μM vs RAP 100 μM	7.6×10^{-4}

3.1.3 Broad-range dose-response (1, 10, 50, and 100 μM) with matching Vehicles

To determine whether the effect of the higher rapamycin concentration (n.s from Vehicle) is driven by rapamycin (RAP) or the DMSO vehicle, each RAP concentration was matched with its vehicle concentrations (0.01%, 0.1%, 0.5%, and 1%), alongside an untreated control (Figure 13). This experiment was designed as a short-duration rapid screen of rapamycin versus its DMSO vehicle and is interpreted in that context. Across all rapamycin concentrations, treatment significantly improved paralysis relative to untreated worms ($p = 3 \times 10^{-5}$). The protection effect observed at

the lowest and highest concentrations: RAP 1 μ M reduced paralysis events in comparison with untreated worms ($p = 0.027$), and RAP 100 μ M further reduced the paralysis ($p = 1.7 \times 10^{-4}$). RAP 10 μ M and RAP 50 μ M concentrations showed intermediate trends but did not reach statistical significance after Bonferroni correction ($p = 0.098$ and $p = 0.155$, respectively).

Rapamycin was delivered in DMSO, therefore, each concentration was paired with a matched vehicle control (0.01%, 0.1%, 0.5% and 1%) to isolate the pharmacological contribution of the drug. At low DMSO concentrations, the vehicle alone did not differ from untreated animals (DMSO 0.01%, $p = 0.429$ vs untreated; DMSO 0.1%, $p = 0.740$) (Table 2). By eliminating the effect of DMSO, RAP 1 μ M remained significantly protective relative to untreated ($p = 0.0081$) and trended toward improved survival relative to its matched vehicle ($p = 0.120$), and RAP 10 μ M was likewise significantly better than untreated ($p = 0.030$) without a corresponding vehicle effect. These comparisons indicate that the protective effect at 1 and 10 μ M rapamycin is attributable to the drug itself rather than to the DMSO vehicle.

At higher DMSO concentrations, however, the vehicle itself had an effect on A β -induced paralysis. DMSO 0.5% differed from untreated ($p = 0.023$) and DMSO 1% produced strong protection on its own ($p = 0.0027$ vs untreated). Pairwise comparisons within the DMSO-only analysis confirmed that DMSO 1% differed significantly from both DMSO 0.01% ($p = 0.042$) and DMSO 0.1% ($p = 0.024$), establishing a dose-dependent vehicle effect. Consequently, the apparent rescue at RAP 50 μ M and RAP 100 μ M was not statistically different from the protection provided by the matched DMSO vehicle alone (RAP 50 μ M vs DMSO 0.5%, $p = 1.000$; RAP 100 μ M vs DMSO 1%, $p = 0.557$). In other words, although both 50 and 100 μ M rapamycin produced strong absolute improvements in paralysis relative to untreated animals, the contribution of rapamycin above and beyond that of its high-DMSO vehicle could not be resolved in this experiment.

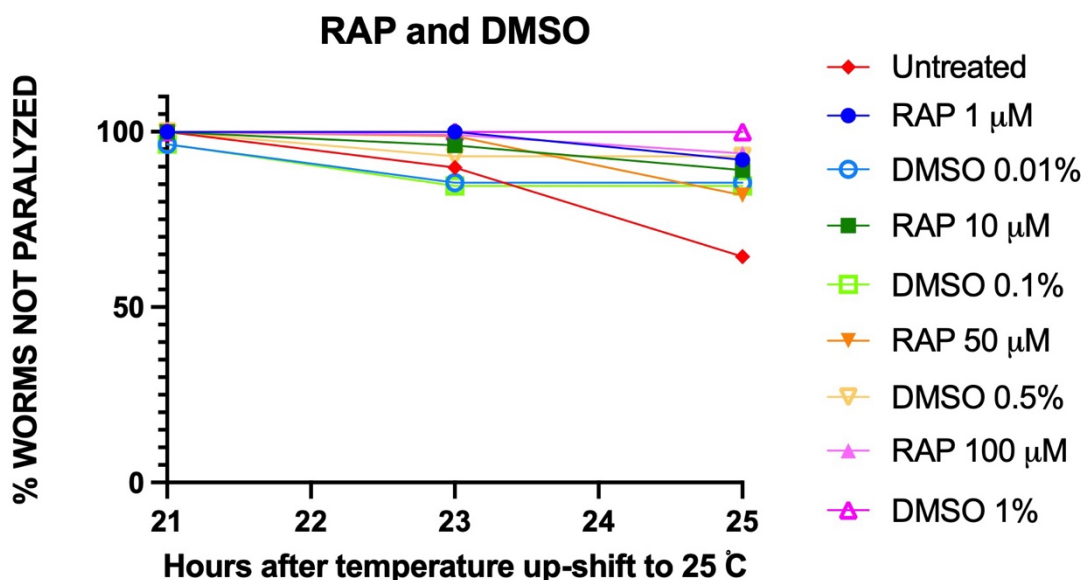


Figure 13. Broad-range rapamycin dose-response with matched DMSO vehicle controls. CL4176 animals were treated with rapamycin (RAP; 1, 10, 50, or 100 μM) or the corresponding DMSO vehicle concentrations (0.01%, 0.1%, 0.5%, or 1%) and scored for paralysis after temperature upshift to 25°C. Overall, rapamycin treatment significantly reduced paralysis compared with untreated worms ($p = 3 \times 10^{-5}$). Beginning at 20 h post-induction, paralysis was scored every 2 h until all vehicle-treated animals were paralyzed. Data are plotted as the percentage of non-paralyzed worms over time; ≥ 50 worms per condition, $n = 1$ independent biological replicate(s). Pairwise comparisons between each Rapamycin concentration and Vehicle were performed by log-rank test in R and curves were generated in GraphPad Prism.

Table 2. Pairwise log-rank comparisons for the low-dose rapamycin matched-vehicle rapid screening test shown in Figure 13. Each rapamycin (RAP) concentration (1, 10, 50, 100 μM) was compared against the untreated control and against its individually matched DMSO vehicle (0.01%, 0.1%, 0.5%, 1% v/v, respectively). At low DMSO concentrations (0.01% and 0.1%), the vehicle did not significantly differ from untreated controls, confirming that protective effects at 1 and 10 μM rapamycin were drug-mediated. At higher DMSO concentrations (0.5% and 1%), the vehicle itself produced a significant effect, indicating that apparent rescue at 50 and 100 μM rapamycin was primarily attributable to the DMSO carrier. p-values reported as Bonferroni-adjusted log-rank test results.

Condition	vs Untreated (p)	vs matched DMSO (p)
RAP 1 μM	0.027	0.120
DMSO 0.01%	0.429	—
RAP 10 μM	0.030	0.390
DMSO 0.1%	0.740	—
RAP 50 μM	0.046	1.000

Condition	vs Untreated (p)	vs matched DMSO (p)
DMSO 0.5%	0.023	—
RAP 100 μ M	1.7×10^{-4}	0.557
DMSO 1%	0.0027	—

3.1.4 Longer term study focuses on low-dose rapamycin

To test whether the low-dose effects observed in Section 3.1.3 reflected a genuine pharmacological action of rapamycin, a third round of paralysis assays was performed in which 1 μ M and 10 μ M rapamycin were compared against the matched DMSO concentrations (0.01% and 0.1% v/v, respectively) (Table 3). The resulting paralysis curves are shown in (Figure 14).

All four conditions (1 μ M rapamycin, 0.009% DMSO, 10 μ M rapamycin, and 0.09% DMSO) produced essentially identical paralysis trajectories across the entire 20–35 h scoring window. This result confirms that, at concentrations of 10 μ M and below, rapamycin exert no measurable protective effect against A β -induced paralysis in CL4176 and demonstrates that the apparent 1- and 10- μ M signals seen in the broad-range scan (Figure 11, Table 2) was diminished with longer term experiment. The 1–10 μ M range was therefore eliminated from further consideration as a working dose, and the dose-finding effort was redirected toward concentrations that is >10 μ M and <50 μ M, we chose to start between 20 and 30 μ M, immediately above the no-effect ceiling and below the upper bound of the original scan.

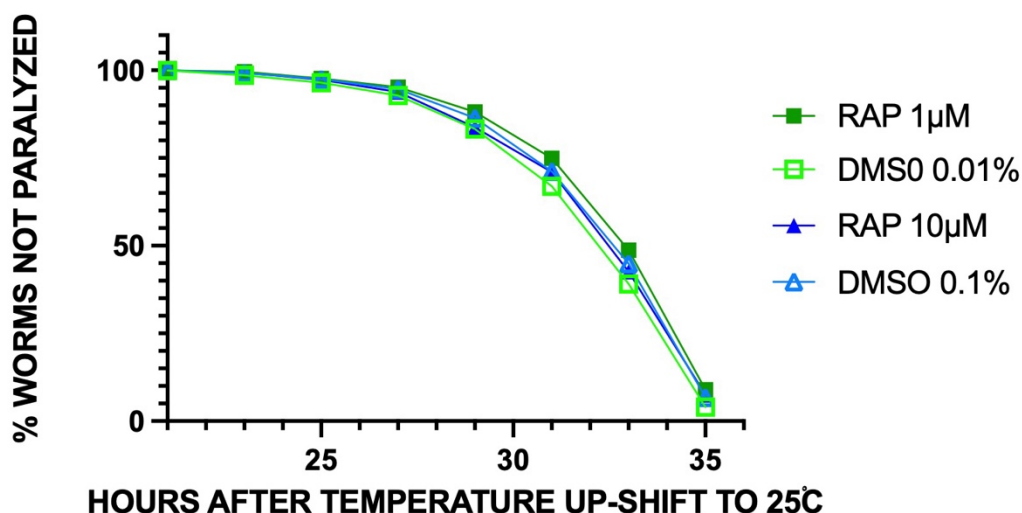


Figure 14. Extended paralysis assay demonstrates no sustained protective effect of low-dose rapamycin. CL4176 worms expressing human A β ₁₋₄₂ in body-wall muscle were treated with rapamycin (RAP; 1 or 10 μ M) or the corresponding DMSO vehicle controls (0.01% and 0.1% v/v, respectively). Beginning at 20 h post-induction, paralysis was scored every 2 h until all vehicle-treated animals were paralyzed. Data are plotted as the percentage of non-paralyzed worms over time; ≥ 50 worms per condition, n = 1 independent biological replicate(s). Pairwise comparisons between each Rapamycin concentration and Vehicle were performed by log-rank test in R and curves were generated in GraphPad Prism.

Table 3. Pairwise log-rank comparisons for the extended low-dose rapamycin versus matched-vehicle assay shown in Figure 14. CL4176 worms treated with rapamycin (RAP, 1 or 10 μ M) or matched DMSO vehicle (0.01% or 0.1% v/v) were compared across all pairwise combinations. All four conditions produced statistically indistinguishable paralysis trajectories in the extended observation window, confirming absence of pharmacological activity at concentrations ≤ 10 μ M. Bonferroni-adjusted log-rank p-values are presented; n.s., not significant =1.

Pairwise comparison	Bonferroni-adjusted p-value
DMSO 0.01% vs DMSO 0.1%	0.03
DMSO 0.01% vs RAP μ M	0.214
DMSO 0.01% vs RAP 10 μ M	4e-05
DMSO 0.1% vs RAP 1 μ M	n.s.
DMSO 0.1% vs RAP 10 μ M	0.416

RAP 1 μ M vs 10 μ M	0.078
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3.1.5 Narrow-range comparison: 20 μ M and 30 μ M versus matched DMSO

In the fourth round of dose-finding, CL4176 animals were assayed in five conditions: untreated, 20 μ M rapamycin, 0.2% DMSO (vehicle-matched to 20 μ M), 30 μ M rapamycin, and 0.3% DMSO (vehicle-matched to 30 μ M). The paralysis curves are shown in (Figure 15). By contrast with the earlier broad-range scan, this round revealed clear and biologically meaningful separation between drug- and control-treated animals. Both 20 μ M and 30 μ M rapamycin produced visibly delay paralysis curves in comparison to the untreated baseline, with the difference becoming pronounced from approximately 29 hours post-upshift onward. By the final scored timepoint (33 h), untreated animals had reached approximately 40% non-paralyzed, the two DMSO controls reached approximately 55%, and the two rapamycin conditions retained approximately 75–80% non-paralyzed.

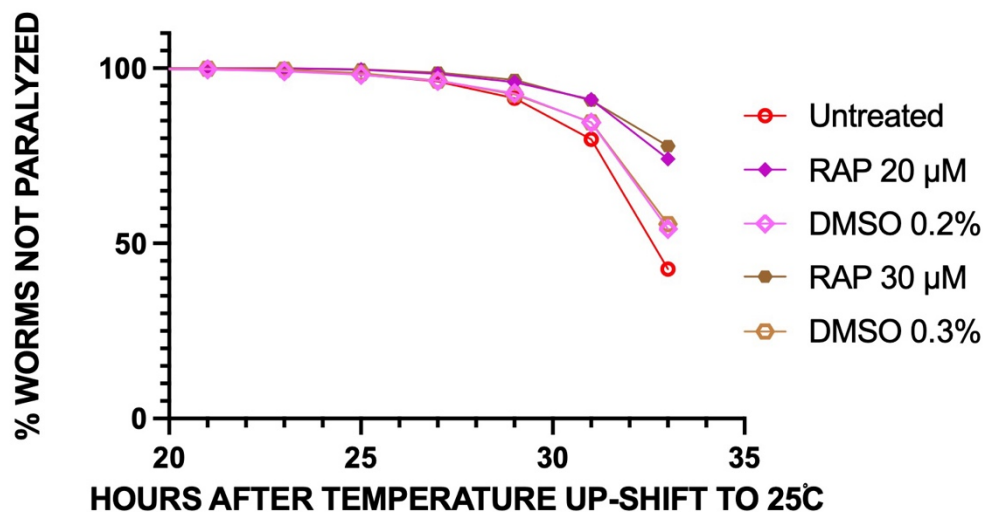


Figure 15. Intermediate-dose rapamycin screen identifies a protective effect at 20–30 μ M with minimal vehicle interference. CL4176 worms expressing human A β_{1-42} in body-wall muscle were treated with rapamycin (RAP; 20 or 30 μ M) or the corresponding DMSO vehicle controls (0.2% and 0.3% v/v,

respectively), alongside an untreated control. Beginning at 20 h post-induction, paralysis was scored every 2 h until all vehicle-treated animals were paralyzed. Data are plotted as the percentage of non-paralyzed worms over time; ≥ 50 worms per condition, $n = 1$ independent biological replicate(s). Pairwise comparisons between each Rapamycin concentration and Vehicle were performed by log-rank test in R and curves were generated in GraphPad Prism.

Table 4. Bonferroni-adjusted pairwise log-rank p-values for the intermediate-dose rapamycin screen shown in Figure 15. All five conditions (untreated, RAP 20 μ M, DMSO 0.2%, RAP 30 μ M, DMSO 0.3%) were compared pairwise by log-rank test with Bonferroni correction. Both 20 μ M and 30 μ M rapamycin were significantly better than their matched DMSO controls ($p = 9.0 \times 10^{-7}$ and $p = 2.1 \times 10^{-7}$, respectively) and were statistically equivalent to one another ($p = 1.000$), confirming a protective plateau at 20 μ M. Both DMSO controls were non-significantly different from untreated ($p = 0.068$ and $p = 0.024$ for 0.2% and 0.3%, respectively), indicating that the vehicle at these concentrations did not substantially confound interpretation; n.s., not significant =1.

	NOT treated	RAP 20 μ M	DMSO 0.2%	RAP 30 μ M
RAP 20 μ M	2.1×10^{-14}	—	—	—
DMSO 0.2%	0.068	9.0×10^{-7}	—	—
RAP 30 μ M	1.1×10^{-15}	n.s.	4.8×10^{-8}	—
DMSO 0.3%	0.024	3.7×10^{-6}	n.s.	2.1×10^{-7}

In Table 4 we shows both 20 μ M and 30 μ M rapamycin produced highly significant protection relative to untreated controls ($p = 2.1 \times 10^{-14}$ and $p = 1.1 \times 10^{-15}$, respectively), confirming that these doses alleviate the induced paralysis. Also that both rapamycin doses were significantly different from their respective DMSO-matched controls (RAP 20 μ M vs DMSO 0.2%, $p = 9.0 \times 10^{-7}$; RAP 30 μ M vs DMSO 0.3%, $p = 2.1 \times 10^{-7}$), formally establishing that the survival benefit was due to the drug treatment rather than to the vehicle. Most importantly for dose selection, the two rapamycin doses were statistically non-significantly different from one another (RAP 20 μ M vs RAP 30 μ M, $p = 1.000$), as were the two DMSO controls (DMSO 0.2% vs DMSO 0.3%, $p = 1.000$).

3.1.6 Selection of 20 μ M as the working concentration

Based on the dose-finding strategy described above, 20 μ M was selected as the working concentration of rapamycin for all subsequent experiments reported in this thesis. Three lines of evidence supported this choice. First, 20 μ M produced a statistically robust reduction of paralysis onset (Figure 15) relative to both the untreated and DMSO-matched controls, whereas concentrations of 10 μ M and below were indistinguishable from vehicle (Figure 14). Second, 20 μ M treatment has an identical curve, with no measurable additional benefit gained at 30 μ M ($p = 1.000$), indicating that this concentration was sufficient to provide the protective phenotype under the present assay conditions. Third, selecting the lowest effective concentration minimized both the absolute amount of rapamycin used and the corresponding DMSO load (0.2% versus 0.3% v/v at 30 μ M).

3.2 Rapamycin Delays A β -Induced Paralysis in CL4176

CL4176 strain expresses human A β_{1-42} in body-wall muscle under control of a temperature-sensitive promoter, and the upshift from 16 °C to 25 °C drives the rapid accumulation of A β proteins and a paralysis phenotype. Synchronized L1 larvae were transferred onto NGM plates seeded with OP50 and supplemented with either 0.2% DMSO (vehicle) or 20 μ M rapamycin, conditioned for 48 hours at 16 °C, and then upshifted to 25 °C to induce transgene expression. Paralysis was scored at fixed intervals from 20 hours post-temperature shift until all worms are paralyzed, and the proportion of non-paralyzed animals at each timepoint was used to construct the curves shown in Figure 16.

Rapamycin 20 μ M paralysis assay

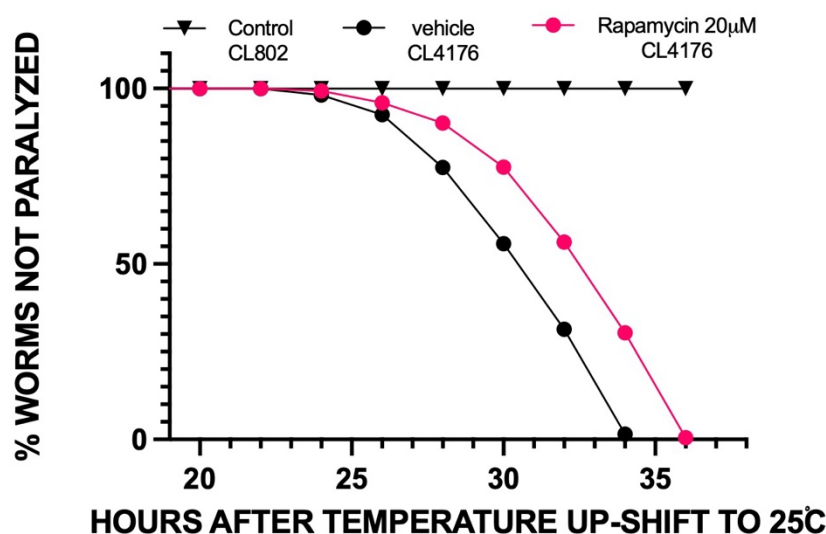


Figure 16. Rapamycin (20 μ M) significantly delays A β -induced paralysis in CL4176 worms. CL4176 worms expressing human A β ₁₋₄₂ in body-wall muscle were treated with 20 μ M rapamycin or the matched DMSO vehicle control (0.2% v/v). Beginning at 20 h post-induction, paralysis was scored every 2 h until all vehicle-treated animals were paralyzed. Data are plotted as the percentage of non-paralyzed worms over time; ≥ 50 worms per condition, $n = 1$ independent biological replicate(s). Pairwise comparisons between each Rapamycin concentration and Vehicle were performed by log-rank test in R and curves were generated in GraphPad Prism.

Vehicle-treated CL4176 animals displayed the expected A β -induced paralysis trajectory, with a steep decline in motility beginning at approximately 25 hours post-upshift and the majority of the population paralyzed by 32 hours. On the other hand, Rapamycin-treated animals retained motility for substantially longer: the paralysis curve was delayed at every measured timepoint, and the difference between the two curves was already evident by the 25-hour interval while no effect detected on the transgenic control strain CL802. Pairwise Log-rank comparison of the 3 curves with Bonferroni correction for multiple testing yielded a highly significant difference between groups ($p = 0.0017$), confirming that the paralysis progression was delayed by drug treatment.

To quantify the magnitude of this delay, the time at which 50% of the population was paralyzed (PT_{50}) was calculated as follows: $(PT_{50}) = t_1 + (p_2 - p_1)(t_2 - t_1)$.

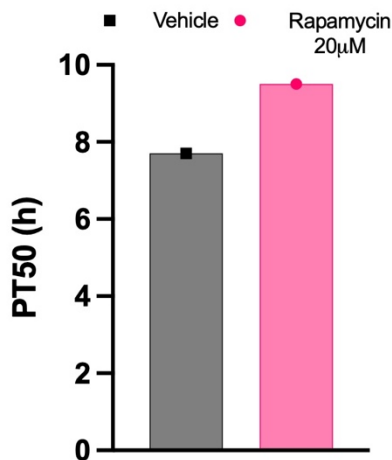


Figure 17. Rapamycin increases PT₅₀ in transgenic *C. elegans*. Bar graph showing the paralysis time at 50% (PT₅₀) for worms treated with vehicle or 20 µM Rapamycin. Vehicle-treated animals reached a PT₅₀ of 7.7 h, whereas rapamycin-treated animals reached 9.5 h, indicating that rapamycin delays paralysis onset. ≥ 50 worms per condition, $n = 1$ independent biological replicate(s).

As summarized in (Figure 17), vehicle-treated CL4176 animals reached PT₅₀ at 7.7 hours after the onset of scoring, whereas rapamycin-treated animals reached PT₅₀ at 9.5 hours, corresponding to a 1.8-hour (~23%) extension of the time of paralysis progression. The consistency of the drug effect across the entire paralysis trajectory suggests that 20 µM rapamycin slows the underlying rate of A β accumulation and the improvement of locomotor dysfunction rather than simply delaying its onset. Together, these data establish that rapamycin provides measurable protection against acute A β proteotoxicity in body-wall muscle.

3.3 Rapamycin Improves Chemotactic Index in A β -Expressing CL2355

Whereas the paralysis assay reports muscle-driven A β toxicity, the chemotaxis assay examines the consequences of pan-neuronal A β expression on neurosensory function and the possibility of rescue with treatment. CL2355 animals express human A β_{1-42} in their neurons leading to a loss of

attraction to volatile odorants such as benzaldehyde, an established attractant for A β -related neurosensory impairment in this model. CL2122, the strain-matched non-A β transgenic control, was assayed in parallel with CL2355 to distinguish A β -specific rescue from non-specific behavioral effects of rapamycin. Because CL2122 retains intact chemotaxis after the same temperature-induction protocol used to induce A β expression in CL2355, any improvement in Chemotaxis Index (CI) seen in CL2355 but not in CL2122 was interpreted as a treatment effect on A β -driven proteotoxicity rather than on locomotion, olfaction, or general behavior. Following the conditioning protocol summarized in (Figure 9) (36 h at 16 °C followed by 36 h at 25 °C on vehicle- or rapamycin-supplemented plates), age-synchronized day-1 adults were washed, placed within the chemotaxis boundaries, and allowed to distribute between the test (T) and control © quadrants for one hour. The chemotaxis index ($CI = (T - C)/(T + C)$) was then computed for each plate.

Chemotaxis assay using Rapamycin 20 μ M

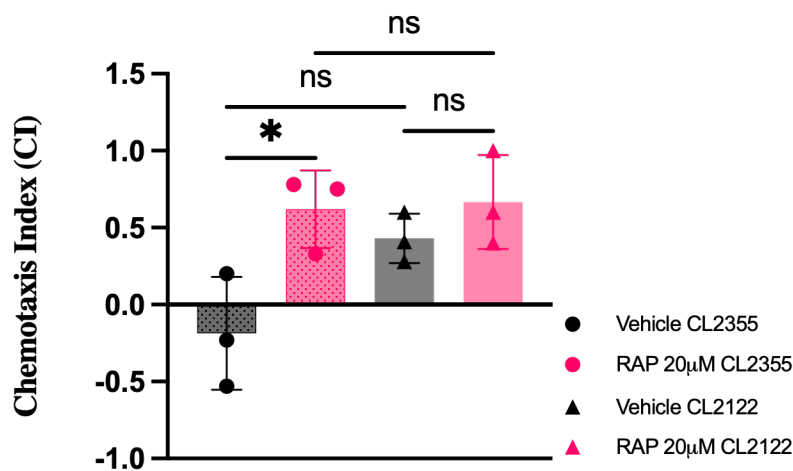


Figure 18. Effect of Rapamycin on chemotaxis behavior in non-A β and A β *C. elegans* strains. Chemotaxis Index (CI) was quantified in CL2355 (A β -expressing) and CL2122 (non-A β) animals treated with vehicle or 20 μ M Rapamycin (RAP). Rapamycin produced an increase in CI in both strains and appeared to rescue the impaired chemotaxis phenotype of CL2355. Statistical analysis with one-way

ANOVA gave $p=0.015$ between Rapamycin and vehicle in transgenic strain whereas no significance shown in the control strain. 50-65 worms per condition. $N= 3$.

As shown in (Figure 18), vehicle-treated CL2355 animals displayed an impaired chemotactic response, with a mean CI close to zero, consistent with the reported neuronal A β phenotype of this strain. Following treatment, 20 μ M rapamycin produced a clear improvement in the CL2355 chemotactic system, restoring the CI toward values approaching those of the non-transgenic controls. Multiple-Comparisons test confirmed that the difference between rapamycin- and vehicle-treated CL2355 animals was statistically significant ($p = 0.015$), indicating a genuine rescue of the A β -induced behavioral deficit. On the other hand, CL2122 animals showed no significant difference in CI between vehicle and rapamycin conditions (n.s.), and their already-high baseline CI was largely preserved under both treatments. This selectivity, efficacy in the proteotoxic strain, but no measurable change in the control, indicates that the behavioral improvement is attributable to mitigation of A β pathology rather than to a non-specific stimulant or sensory effect of the drug.

3.4 Rapamycin Enhances Crawling Locomotion Parameters

To determine the qualitative motility changes observed in the A β -muscle strain paralysis assay into quantitative neuromuscular metrics, crawling locomotion was independently quantified on solid NGM. Four parameters were measured: peristaltic speed (Figure 19A) (the rate of forward translation produced by body-wave propagation), mobility idle time (Figure 19B) (the cumulative duration spent stationary during the recording window), center-point speed (Figure 19C) (the displacement of the animal's centroid over time), wavelength (Figure 19D) (the spatial period of the body wave).

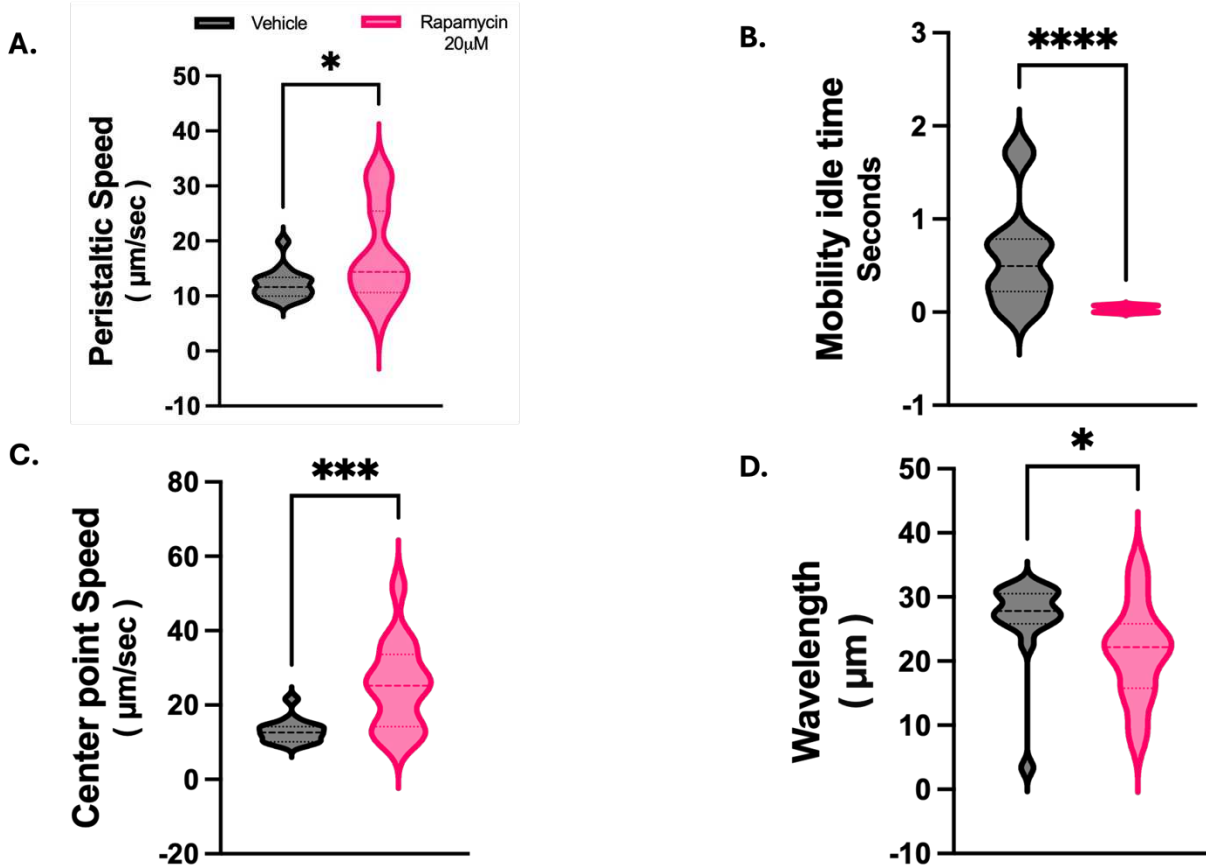


Figure 19. Rapamycin alters crawling locomotion parameters in CL4176 transgenic *C. elegans* following $A\beta_{1-42}$ induction. Quantitative analysis of WormLab-tracked crawling behavior in age-synchronized CL4176 worms cultured at 16 °C on NGM containing either 0.2 % DMSO (Vehicle, black/grey) or 20 μ M Rapamycin (magenta). Videos were imported into WormLab software (MBF Bioscience) for automated worm detection and tracking. **(A)** Peristaltic speed (μ m/s). Rapamycin-treated worms displayed a significantly higher peristaltic speed than Vehicle-treated worms ($p < 0.05$, *), **(B)** mobility idle time. Rapamycin-treated worms displayed a significantly higher peristaltic speed than Vehicle-treated worms ($p < 0.0005$, ****), **(C)** Center point speed (μ m/s). Rapamycin-treated worms displayed a significantly higher peristaltic speed than Vehicle-treated worms ($p < 0.0005$, ***). **(D)** Wavelength (μ m). Rapamycin-treated worms displayed a significantly higher peristaltic speed than Vehicle-treated worms ($p < 0.05$, *). Violin plots show the distribution of individual worm tracks, with the median (dashed line) and quartiles (dotted lines) indicated. Each violin represents pooled data from $n = 1$ independent biological replicate (≥ 50 worms per replicate per condition). Statistical comparisons between Vehicle and Rapamycin were performed using an unpaired two-tailed Student's *t*-test in GraphPad Prism. Significance is indicated as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant.

Rapamycin-treated worms showed a significant increase in peristaltic speed compared with vehicle controls (Mann–Whitney test, $p < 0.05$), indicating more effective conversion of body-wave

activity into forward locomotion. Center-point speed was significantly enhanced ($p < 0.001$), with the rapamycin distribution shifted upward and the inter-animal variance reduced relative to vehicle. Wave wavelength was also significantly affected ($p < 0.001$). Mobility idle time was profoundly reduced in rapamycin-treated animals ($p < 0.0001$) in comparison to the substantial idle periods observed in vehicle controls.

3.5 Rapamycin Improves Swimming

Because swimming and crawling are regulated by partially distinct neural circuits, swimming locomotion of A β -muscle strain CL4176 was analyzed in vehicle- and rapamycin-treated animals using video tracking. Four parameters were examined a per-animal basis: wave initiation rate (a measure of the frequency at which propulsive body waves are generated at the head/tail), swimming speed ($\mu\text{m}\cdot\text{s}^{-1}$), dynamic amplitude (the magnitude of body bends executed during swimming), and wave attenuation (the degree to which a wave decays as it propagates from head to tail).

The results are presented as violin plots in (Figure 20), with the median indicated. Rapamycin treated animals exhibited a significant increase in wave initiation rate compared with vehicle controls (Mann–Whitney test, $p < 0.05$), indicating that the drug enhances the central pattern generating activity that drives undulatory locomotion. Swimming speed was likewise significantly elevated by rapamycin treatment ($p < 0.001$), with the upper tail of the distribution extending well beyond the vehicle range; this is consistent with both improved wave generation and more efficient force transmission to the surrounding fluid. Dynamic amplitude was significantly increased ($p < 0.01$), demonstrating that treated animals produced larger-amplitude body bends and were therefore not merely thrashing more rapidly with shallower waveforms. Finally, wave attenuation

was significantly altered ($p < 0.05$), with rapamycin-treated animals showing values closer to zero than vehicle-treated animals; this indicates that propulsive waves were better preserved as they travelled along the body, a hallmark of healthy neuromuscular coupling.

The convergence of crawling (Figure 19) and swimming (Figure 20) improvements provides robust evidence that rapamycin preserves neuromuscular function in this model. Importantly, the reduction in idle time indicates that treated animals are not only capable of better movement when active but also spend more of their time engaged in coordinated locomotor behavior, an outcome relevant to the broader concept of health-span extension.

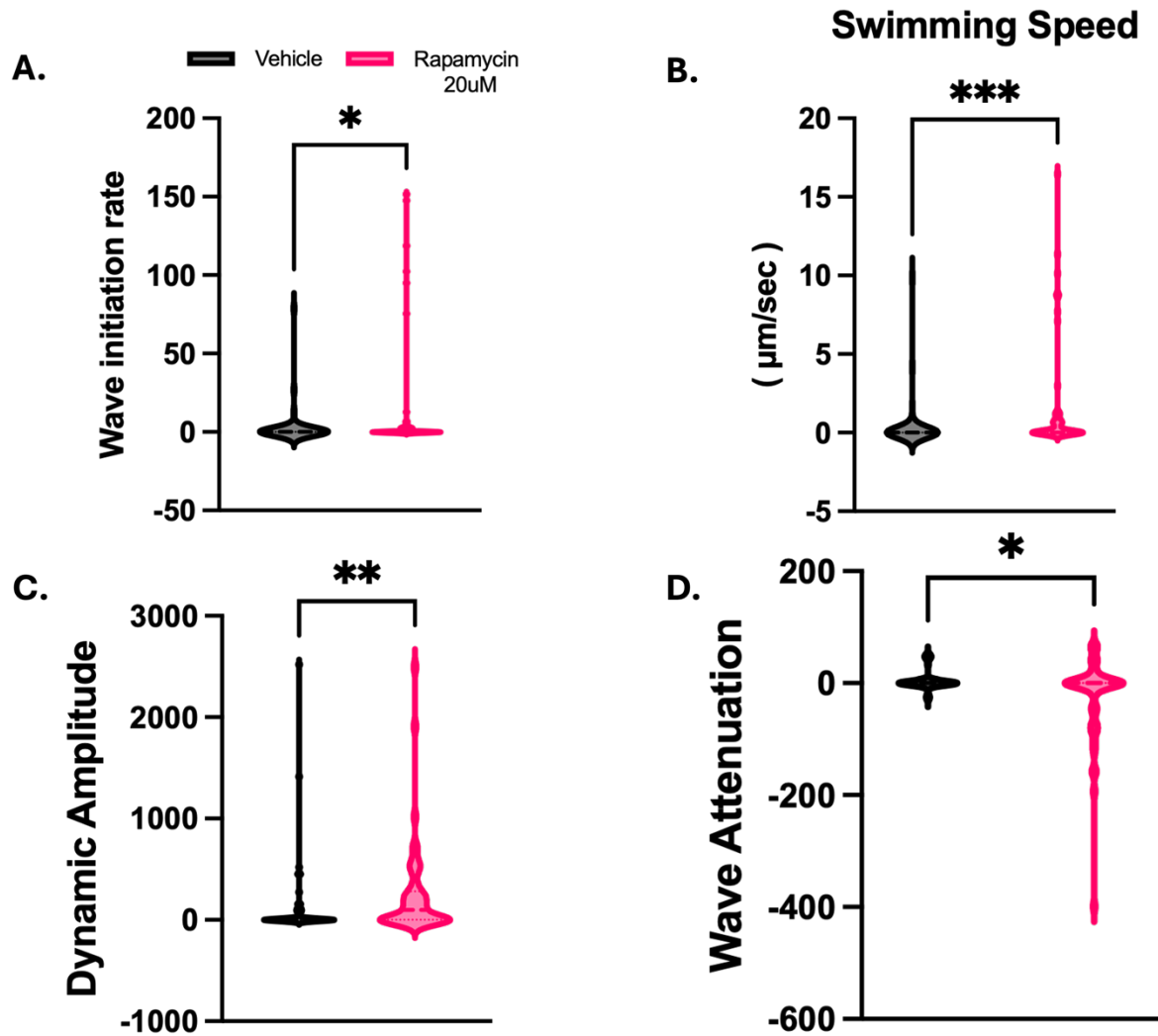


Figure 20. Rapamycin improves swimming locomotion parameters in CL4176 *C. elegans*. Quantitative analysis of swimming locomotion in CL4176 worms treated with vehicle or 20 μM rapamycin following $\text{A}\beta_{1-42}$ induction. Parameters examined: (A) wave initiation rate ($p < 0.05$), (B) swimming speed ($\mu\text{m/s}$; $p < 0.001$), (C) dynamic amplitude (μm ; $p < 0.01$), and (D) wave attenuation (AU; $p < 0.05$). Violin plots show distribution of individual worm tracks. Rapamycin-treated animals showed significant improvements across all four swimming parameters, providing circuit-independent evidence for preserved neuromuscular function. Statistical comparisons by unpaired Mann–Whitney test. Significance: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ≥ 50 worms per condition, $n = 1$ biological replicate.

3.6 Rapamycin Extends Lifespan in A β -Expressing *C. elegans*

Finally, to determine whether the acute and behavioral benefits of rapamycin translate into a measurable extension of organismal survival, Kaplan–Meier lifespan analysis was performed in CL2355 (neuronal A β_{1-42}) and the isogenic CL2122 control. Approximately 100 age synchronized animals per condition were maintained in vehicle- or 20 μ M rapamycin supplemented 24 well plate and scored every other day for survival until the entire population had died (Figure 21).

Vehicle-treated CL2355 animals displayed the expected accelerated mortality phenotype relative to vehicle-treated CL2122 controls, with a decreased survival consistent with the cumulative impact of neuronal A β expression on organismal viability. Rapamycin treatment delayed mortality in both genetic backgrounds, with the most pronounced rightward shift observed in the A β -expressing CL2355 strain. Pairwise comparison of all four survival curves using the Log-rank test with Bonferroni correction for multiple comparisons yielded a highly significant overall difference ($p < 2 \times 10^{-16}$), indicating that survival distributions were not drawn from a common underlying lifespan.

The magnitude and consistency of the lifespan extension in CL2355, combined with the parallel improvement observed in CL2122, indicate that rapamycin exerts both a disease-modifying effect (mitigating A β -driven mortality) and an underlying pro-longevity effect that is consistent with its well-established role as an inhibitor of the mTORC1 pathway. It should be noted, however, that these survival data represent a double biological replicate ($N = 2$) of approximately 100 worms per condition; while the within-experiment statistical power is high, biological replication will be required to estimate the inter-experiment variability of the effect.

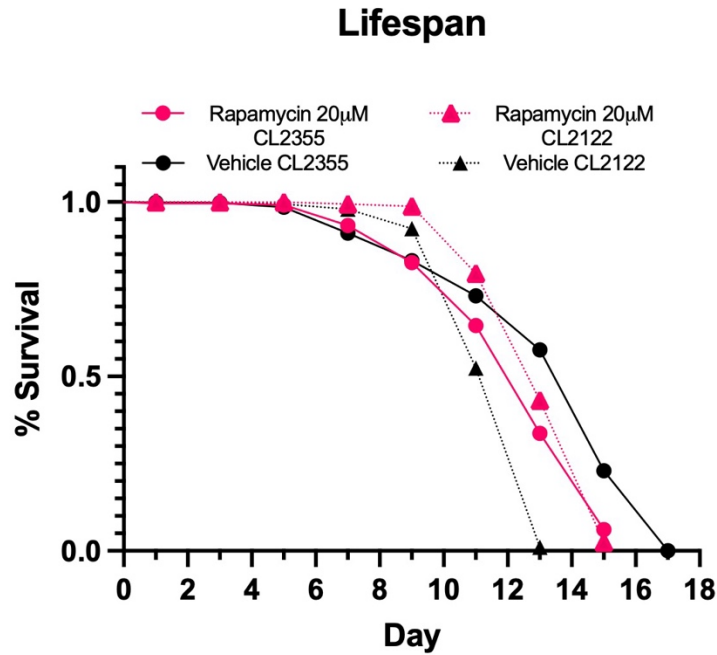


Figure 21. Rapamycin extends lifespan in A β -expressing CL2355 transgenic *C. elegans*. Kaplan–Meier survival curves for CL2355 (pan-neuronal A β_{1-42}) and CL2122 (non-transgenic control) animals treated with vehicle (0.2% DMSO) or 20 μ M rapamycin. Vehicle-treated CL2355 animals displayed accelerated mortality relative to CL2122 controls ($p < 2 \times 10^{-16}$). Rapamycin treatment significantly extended survival in CL2355 animals ($p < 2 \times 10^{-16}$), with complete lethality delayed from day 13 (vehicle) to day 15 (rapamycin). Vehicle- and rapamycin-treated CL2122 survival curves were nearly superimposable ($p = 0.014$). Pairwise Bonferroni-adjusted log-rank p-values are presented in (Table 5). ~100 worms per group, $n = 2$ biological replicates.

Table 5. Pairwise log-rank (Mantel–Cox) comparisons with Bonferroni adjustment for the lifespan assay shown in Figure 21. Survival distributions of CL2355 vehicle, CL2122 vehicle, CL2355 rapamycin, and CL2122 rapamycin were compared pairwise. Rapamycin treatment significantly extended survival in CL2355 animals ($p < 2 \times 10^{-16}$ relative to vehicle CL2355) and significantly reduced survival relative to CL2122 controls (Rapamycin CL2355 vs. Vehicle CL2122, $p = 3.9 \times 10^{-13}$). The marginal difference between CL2122 vehicle and CL2122 rapamycin ($p = 0.014$) indicates a modest longevity effect in the absence of A β under these conditions. ~100 worms per group, $n = 2$ biological replicates.

	Vehicle CL2355	Vehicle CL2122	Rapamycin CL2355
Vehicle CL2122	< 2e-16	-	-
Rapamycin CL2355	< 2e-16	3.9e-13	-
Rapamycin CL2122	< 2e-16	0.014	< 2e-16

3.7 Rapamycin extends lifespan and improves Health-span of neurons

Survival analysis revealed a protective effect of rapamycin specifically in the A β -expressing CL2355 strain (section 3.6). Vehicle-treated CL2355 worms exhibited rapid mortality with complete lethality by day 11 (Figure 22, solid black line). In contrast, rapamycin treatment significantly extended the lifespan of CL2355 worms, with complete lethality delayed until day 13 (Figure 22, solid pink line).

Importantly, the lifespan-extending effects of rapamycin were largely specific to the A β expressing strain. The control CL2122 strain showed minimal mortality over the 14-day observation period, with both vehicle and rapamycin-treated groups maintaining greater than 90% survival through day 10 and declining to approximately 30% survival by day 13 (Figure 22, dotted lines). The survival curves of vehicle- and rapamycin-treated CL2122 worms were nearly superimposable, indicating that rapamycin had minimal effect on lifespan in the absence of A β pathology under these experimental conditions.

Statistical analysis using log-rank (Mantel-Cox) tests confirmed significant differences between treatment groups. Rapamycin treatment significantly improved survival in CL2355 worms compared to vehicle-treated CL2355 controls ($p < 0.05$) with exact pairwise comparisons result after Bonferroni adjustment shown in (Table 6). As expected, vehicle-treated CL2355 worms showed significantly reduced survival compared to vehicle-treated CL2122 controls ($p < 0.001$), confirming the toxic effects of A β expression.

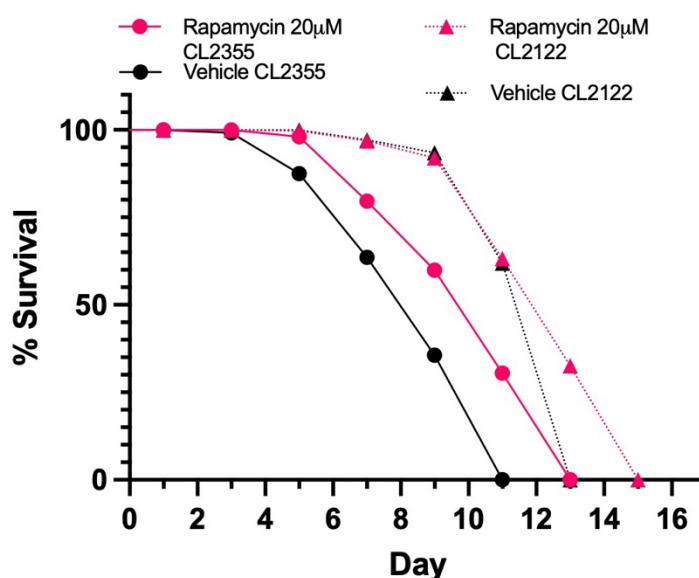


Figure 22. Rapamycin-extended lifespan analysis linked to healthspan evaluation in CL2355 and CL2122. Kaplan–Meier survival curves for CL2355 and CL2122 animals treated with vehicle or 20 μ M rapamycin in the combined survival–locomotion experiment. Survival scoring and locomotion analysis were performed in parallel on the same cohorts. Vehicle-treated CL2355 worms showed complete lethality by day 11; rapamycin-treated CL2355 worms showed delayed complete lethality to day 13. Pairwise Bonferroni-adjusted log-rank p-values are presented in (Table 6). ~100 worms per group, n = 2 biological replicates.

Table 6. Pairwise log-rank comparisons of the combined survival–locomotion assay shown in Figure 22. Survival curves for CL2355 and CL2122 animals treated with vehicle or rapamycin were compared by log-rank test with Bonferroni adjustment. Rapamycin significantly extended survival in CL2355 animals ($p = 0.015$ relative to vehicle CL2355, Bonferroni-adjusted). Vehicle-treated CL2355 animals showed significantly reduced survival relative to vehicle-treated CL2122 controls ($p < 2 \times 10^{-16}$). Rapamycin-treated CL2355 animals showed significantly lower survival than rapamycin-treated CL2122 animals ($p = 8.5 \times 10^{-5}$), confirming residual A β -driven mortality even under drug treatment. Vehicle- and rapamycin-treated CL2122 animals were not significantly different ($p = 1$). ~100 worms per group, n = 2 biological replicates ; n.s., not significant =1.

	Vehicle CL2355	Vehicle CL2122	Rapamycin CL2355
Vehicle CL2122	< 2e-16	-	-
Rapamycin CL2355	< 2e-16	0.015	-
Rapamycin CL2122	< 2e-16	n.s.	8.5e-05

To determine whether rapamycin treatment improves healthspan in addition to extending lifespan, we performed a combined survival-locomotion assay in parallel with the survival analysis. This approach exploits the well-established observation that age-associated decline in motility precedes death in *C. elegans* and provides a sensitive readout of physiological aging that can be uncoupled from chronological lifespan. Surviving worms were video-recorded every other day throughout the experiment, and locomotor activity parameters were quantified using automated tracking software (WormLab version: 2024.1.10). We focused our analysis on the CL2355 strain, where rapamycin showed the most pronounced survival benefits.

At day 1 of adulthood, both vehicle and rapamycin-treated CL2355 worms displayed high wave initiation rate (Figure 23A) with no significant detected in the other hand overall activity scores were also high (Figure 23B) but significance was emerged between rapamycin transgenic and non-transgenic control group. The two treatment groups showed similar baseline activity levels, confirming that rapamycin did not impair motor function in young animals.

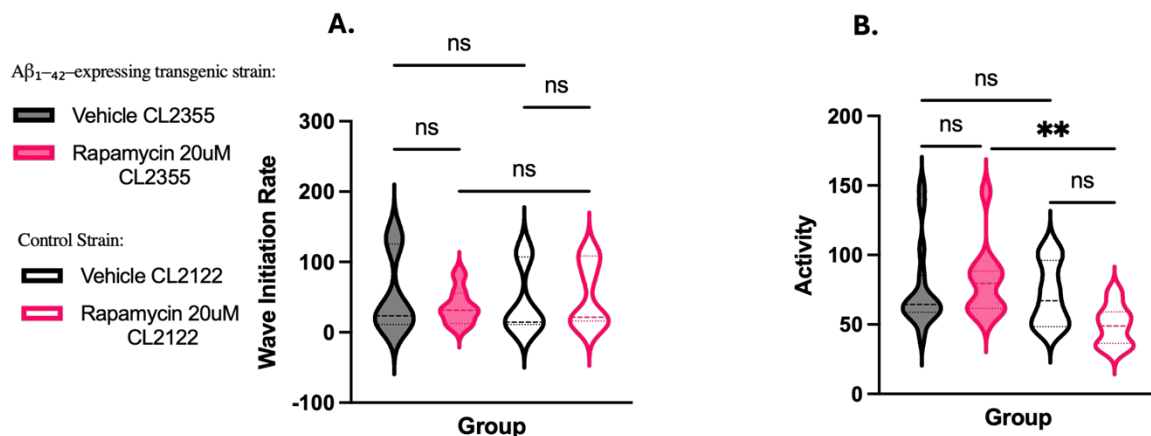


Figure 23. Locomotor activity at Day 1 post-temperature upshift in CL2355 and CL2122. (A) Wave initiation rate and (B) overall activity scores were quantified in CL2355 ($A\beta$) and CL2122 (control) animals

treated with vehicle or 20 μ M rapamycin at Day 1 of adulthood (24 h post-upshift). Both parameters were high across all groups, with no significant between-treatment differences, confirming that rapamycin did not impair motor function in young animals. The elevated Day 1 activity is consistent with an acute locomotor response to the temperature-upshift procedure. Data represent automated WormLab tracking from n = 1 biological replicate.

By day 3, detectable differences in locomotor activity began to emerge between treatment groups (Figure 24). Animals showed a sudden decline in the median and overall distribution of activity scores and wave initiation suggesting the day 1 higher activity with no differences is due to adjustment of the worms following the temperature up-shift. After adjusting to the new environment temperature the change became more pronounced at day 3 (Figure 24), where vehicle-treated CL2355 worms exhibited substantially reduced locomotor activity compared to rapamycin-treated worms. By day 5 the only differences were between the transgenic and control groups of the vehicle while rapamycin transgenic group were insignificant from its control.

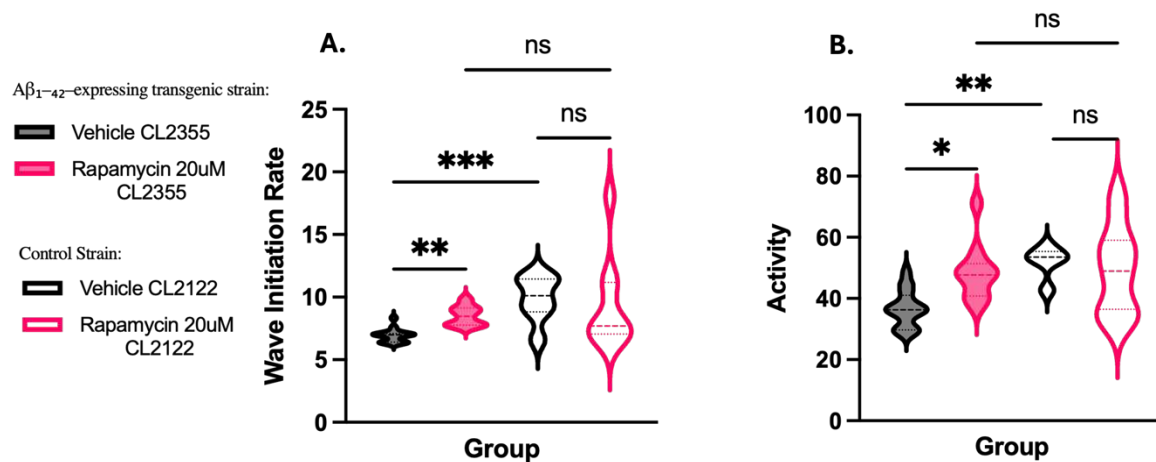


Figure 24. Locomotor activity at Day 3 post-temperature upshift in CL2355 and CL2122. (A) Wave initiation rate and (B) overall activity scores at Day 3. Detectable differences between treatment groups began to emerge as vehicle-treated CL2355 animals showed reduced locomotor activity compared with rapamycin-treated CL2355 animals, reflecting the onset of $A\beta$ -driven neuromuscular decline. Both

parameters showed a decline from Day 1 baseline in all groups, consistent with normalization following the acute temperature-adjustment response. Data from n = 1 biological replicate.

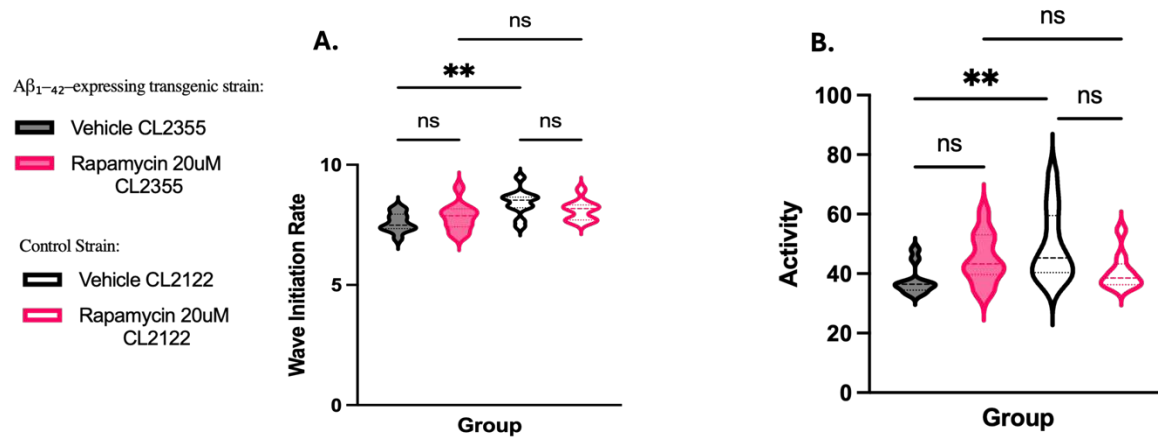


Figure 25. Locomotor activity at Day 5 post-temperature upshift in CL2355 and CL2122. (A) Wave initiation rate and (B) overall activity scores at Day 7. Vehicle-treated CL2355 animals displayed severely compromised motor activity, with many animals exhibiting minimal movement. Rapamycin-treated CL2355 animals retained greater locomotor capacity relative to vehicle-treated CL2355 animals. CL2122 control animals maintained comparatively preserved activity under both treatment conditions. Data from n = 1 biological replicate.

By day 7, vehicle-treated and rapamycin worms displayed severely compromised motor activity, with many animals exhibiting minimal spontaneous movement in the transgenic strain (Figure 26). In contrast, rapamycin-treated worms retained substantial locomotor capacity in the control group.

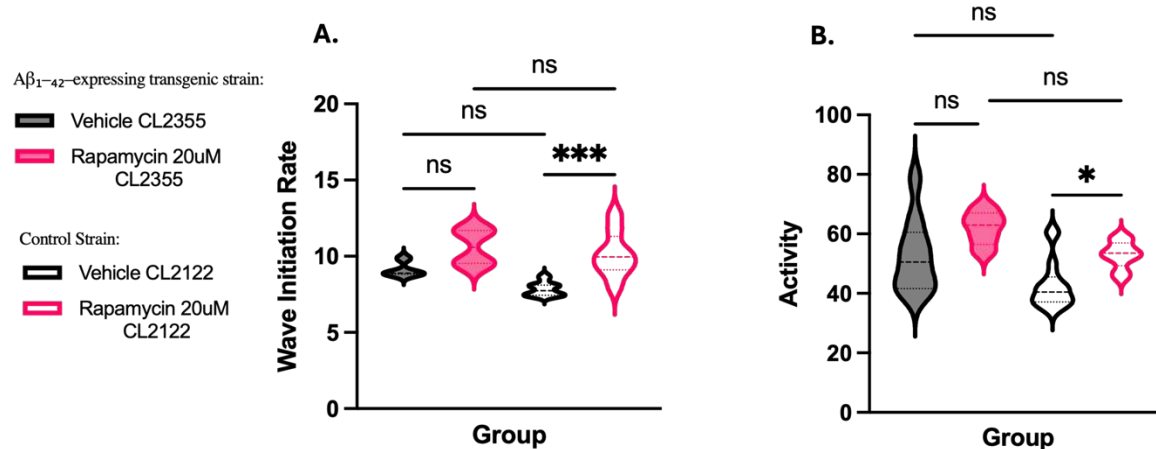


Figure 26. Locomotor activity at Day 7 post-temperature upshift in CL2355 and CL2122. (A) Wave initiation rate and (B) overall activity scores at Day 7. Vehicle-treated CL2355 animals displayed severely compromised motor activity, with many animals exhibiting minimal spontaneous movement. Rapamycin-treated CL2355 animals is not showing rescue relative to vehicle-treated CL2355 animals. CL2122 control animals maintained comparatively preserved activity under both treatment conditions. Data from n = 1 biological replicate.

At day 9 and continuing to day 11, vehicle and rapamycin treated CL2355 worms continued to display deficit locomotor activity, compared to earlier time points.

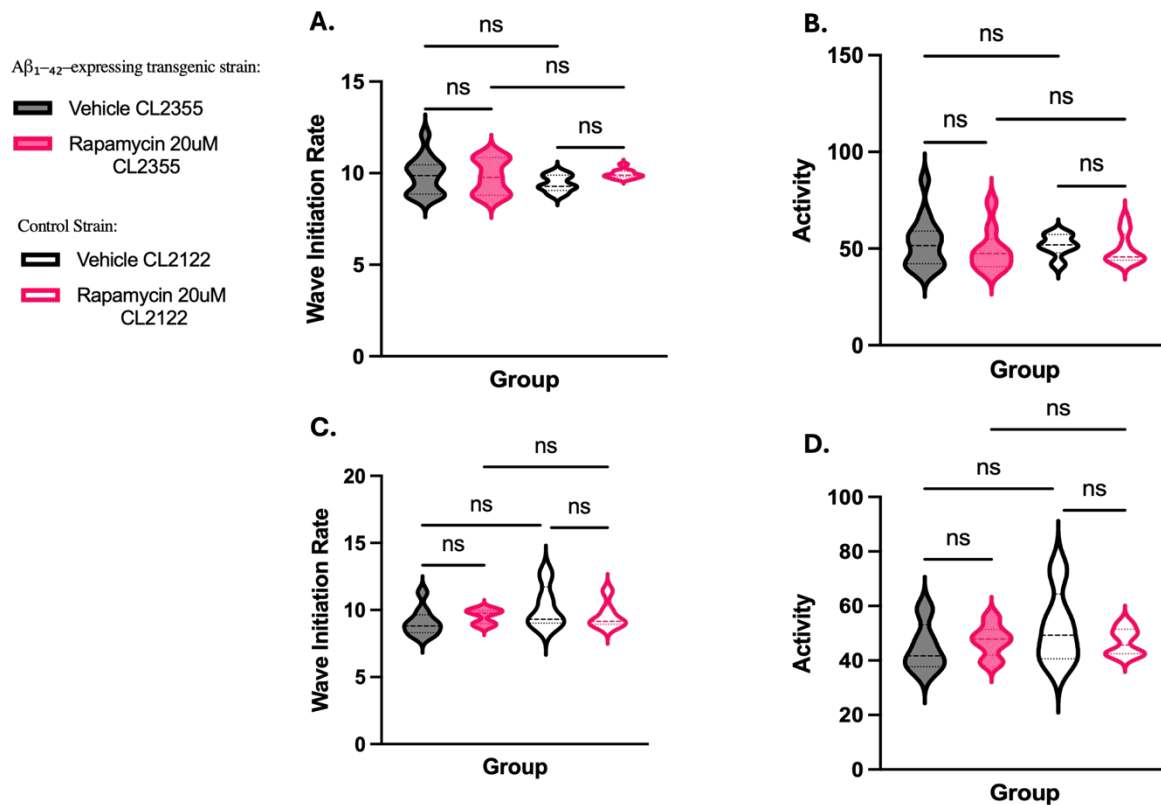


Figure 27. Locomotor activity at Days 9 and 11 post-temperatures upshift in CL2355 and CL2122. (A, C) Wave initiation rate and (B, D) overall activity scores at Days 9 and 11. Both vehicle- and rapamycin-treated CL2355 animals continued to display deficit locomotor activity at late time points compared with earlier assessments, consistent with progressive A β -driven neuronal aging. Data from $n = 1$ biological replicate.

A notable inflection was observed at day 1, where all groups showed temporarily elevated activity (likely reflecting an acute locomotor response to the temperature upshift rather than a treatment effect). To confirm that this initial temperature-adjustment inflation did not drive the statistical interpretation of healthspan, a mixed-effects analysis was performed both including and excluding the day-1 time point. When day 1 was included (Figure 28), no significant main effects of strain, treatment, or their interaction were detected (Table 7), consistent with the day-1 artifact masking underlying differences. Following exclusion of the day-1 time point (Figure 29), the analysis

revealed significant main effects of both strain ($F(1, 159) = 4.493$, $p = 0.0356$) and treatment ($F(1, 159) = 6.950$, $p = 0.0092$), as well as significant strain $\times$ time ($F(2.250, 89.42) = 5.941$, $p = 0.0027$) and treatment $\times$ time ($F(2.250, 89.42) = 2.999$, $p = 0.0489$) interactions (Table 8). These interactions confirm that rapamycin specifically attenuated the trajectory of motor decline in the A β -expressing strain over time.

The temporal alignment of locomotor deterioration with mortality in vehicle-treated CL2355 animals—where severe motor impairment consistently preceded death by one to two days—further confirms that the observed motor differences reflect genuine differences in physiological aging rather than stochastic variation. Rapamycin delayed both the onset of motor decline and progression to lethality, demonstrating that the drug improves healthspan in addition with lifespan extension in the context of A β proteotoxicity.

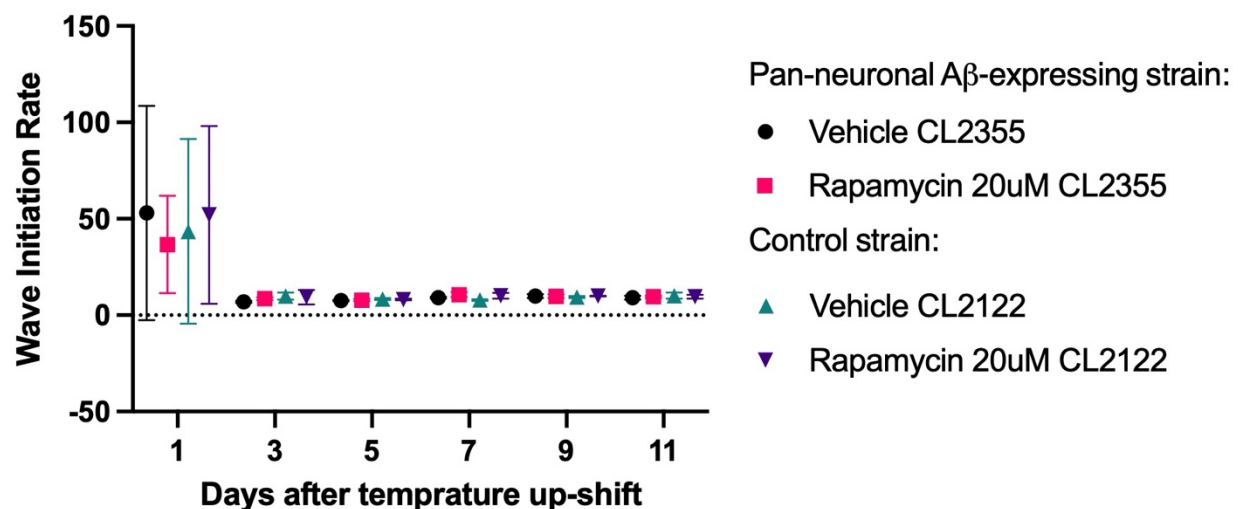


Figure 28. Mixed-effects analysis of wave initiation rate including Day 1 (pre-exclusion). Time-course plot of wave initiation rate across all four groups (CL2355 vehicle, CL2355 rapamycin, CL2122 vehicle, CL2122 rapamycin) at all measured time points including Day 1. The pronounced elevation at Day 1 followed by a sharp drop at Day 3 is consistent with a temperature-adjustment artifact inflating Day 1

values. Statistical results from the mixed-effects model including Day 1 are summarized in (Table 7); no significant main effects of strain, treatment, or their interactions were detected when Day 1 was included.

Table 7. Mixed-effects analysis of wave initiation rate including Day 1 (pre-exclusion). Fixed effects on wave initiation rate across time, strain, and treatment (2×2 design; $n = 50$ subjects, 6 time points including Day 1). No significant main effects of strain ($F(1, 46) = 0.1134$, $p = 0.7378$), treatment ($F(1, 46) = 0.0094$, $p = 0.9232$), or any interaction terms were detected. Only the main effect of time was significant ($F(1.007, 27.59) = 24.89$, $p < 0.0001$), attributable primarily to the Day 1 temperature-adjustment artifact. These results indicate that inclusion of the Day 1 time point masked genuine treatment and strain differences in healthspan ; n.s., not significant =1.

Source of Variation	F (DFn, DFd)	p value	Significance
Time	$F(1.007, 27.59) = 24.89$	< 0.0001	****
Strain	$F(1, 46) = 0.1134$	0.7378	ns
Treatment	$F(1, 46) = 0.0094$	0.9232	ns
Time $\times$ Strain	$F(1.007, 27.59) = 0.0512$	0.8243	ns
Time $\times$ Treatment	$F(1.007, 27.59) = 0.0955$	0.7614	ns
Strain $\times$ Treatment	$F(1, 46) = 0.6008$	0.4423	ns
Time $\times$ Strain $\times$ Treatment	$F(1.007, 27.59) = 0.7359$	0.3993	ns

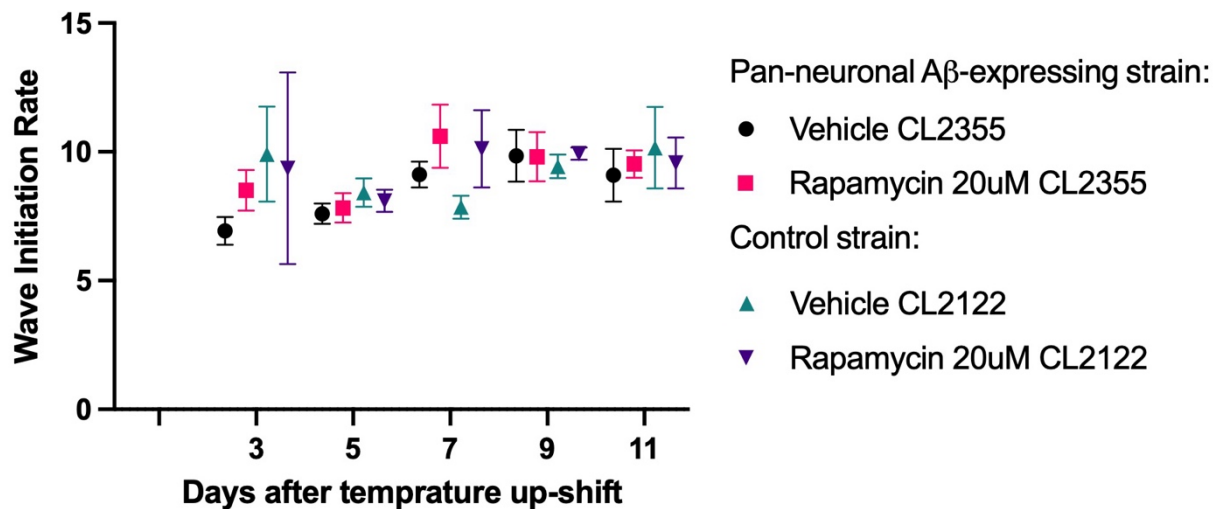


Figure 29. Mixed-effects analysis of wave initiation rate excluding Day 1 (post-exclusion). Time-course plot of wave initiation rate across Days 3–11, following exclusion of the Day 1 temperature-adjustment time point. Exclusion of Day 1 reveals significant main effects of strain ($p = 0.0356$), treatment ($p = 0.0092$), strain $\times$ time interaction ($p = 0.0027$), and treatment $\times$ time interaction ($p = 0.0489$), confirming that rapamycin specifically attenuated the trajectory of motor decline in the A β -expressing CL2355 strain over time. Statistical results are summarized in (Table 8).

Table 8. Mixed-effects analysis of wave initiation rate excluding Day 1 (post-exclusion). Fixed effects on wave initiation rate across time, strain, and treatment (2×2 design; $n = 50$ subjects, 5 time points, Days 3–11). Following exclusion of the Day 1 temperature-adjustment time point, significant main effects of time ($F(2.250, 89.42) = 14.36$, $p < 0.0001$), strain ($F(1, 159) = 4.493$, $p = 0.0356$), and treatment ($F(1, 159) = 6.950$, $p = 0.0092$) were detected, as well as significant strain $\times$ time ($F(2.250, 89.42) = 5.941$, $p = 0.0027$) and treatment $\times$ time ($F(2.250, 89.42) = 2.999$, $p = 0.0489$) interactions. The Strain $\times$ Treatment interaction was not significant ($p = 0.2098$). These results confirm that rapamycin specifically attenuated the trajectory of motor decline in the A β -expressing CL2355 strain over time, independent of the Day 1 inflation artifact; n.s., not significant =1.

Source of Variation	F (DFn, DFd)	p value	Significance
Time	$F(2.250, 89.42) = 14.36$	< 0.0001	****
Strain	$F(1, 159) = 4.493$	0.0356	*
Treatment	$F(1, 159) = 6.950$	0.0092	**
Time $\times$ Strain	$F(2.250, 89.42) = 5.941$	0.0027	**
Time $\times$ Treatment	$F(2.250, 89.42) = 2.999$	0.0489	*
Strain $\times$ Treatment	$F(1, 159) = 1.586$	0.2098	ns
Time $\times$ Strain $\times$ Treatment	$F(2.250, 89.42) = 1.991$	0.1370	ns

The combined survival and locomotion data reveal that rapamycin treatment not only extends chronological lifespan in A β -expressing worms but also delays the onset and progression of age-related motor decline. The temporal trajectory of locomotor deterioration closely tracked with mortality in vehicle-treated animals, with severe motor impairment preceding death by 1-2 days. In rapamycin-treated worms, both the onset of motor decline and the progression to lethality were substantially delayed, indicating that the drug preserves physiological function rather than simply prolonging a moribund state.

These findings demonstrate that rapamycin treatment significantly extends both lifespan and healthspan in an animal model of A β proteotoxicity. The strain-specific effect of rapamycin,

wherein survival and locomotor benefits were observed primarily in the A β -expressing CL2355 strain, suggests that rapamycin specifically ameliorates pathology associated with amyloid aggregation rather than extending lifespan through general longevity mechanisms. The preservation of motor function alongside extended survival indicates that rapamycin maintains neuromuscular integrity in the face of chronic A β toxicity. Collectively, these data support the therapeutic potential of rapamycin for neurodegenerative diseases characterized by protein misfolding and aggregation, including Alzheimer's disease.

3.8 Thioflavin S Staining Does Not Detect Significant Differences in Amyloid- β deposits Between Groups

To assess the effect of rapamycin on amyloid- β (A β) aggregation in the pan-neuronal *C. elegans* model, Thioflavin S (ThS) fluorescence staining was performed twice on three groups: the non-transgenic control strain CL2122 (n = 19), CL2355 worms treated with vehicle (n = 24), and CL2355 worms treated with rapamycin (n = 18). A fixed rectangular region of interest (ROI) was applied consistently to the head region of each worm, and mean fluorescence intensity was measured using ImageJ following a standardized threshold. Individual replicate analyses demonstrated no variability between groups (Figure 25). No statistically significant differences in ThS fluorescence intensity were detected between CL2122 controls, vehicle-treated CL2355, or rapamycin-treated CL2355 animals. The absence of a detectable difference between transgenic and non-transgenic animals suggests that ThS fluorescence under the imaging conditions used was not sufficiently sensitive to resolve the A β deposit burden in the pan-neuronal CL2355 head region, or that A β deposits in this strain do not reach the fibrillar density required for robust ThS reactivity within the experimental time window. This result does not exclude the possibility that rapamycin reduces A β aggregation but indicates that ThS staining under the current protocol is not a reliable

read-out of amyloid burden in this strain. Biochemical approaches were therefore employed to address A β protein-level changes.

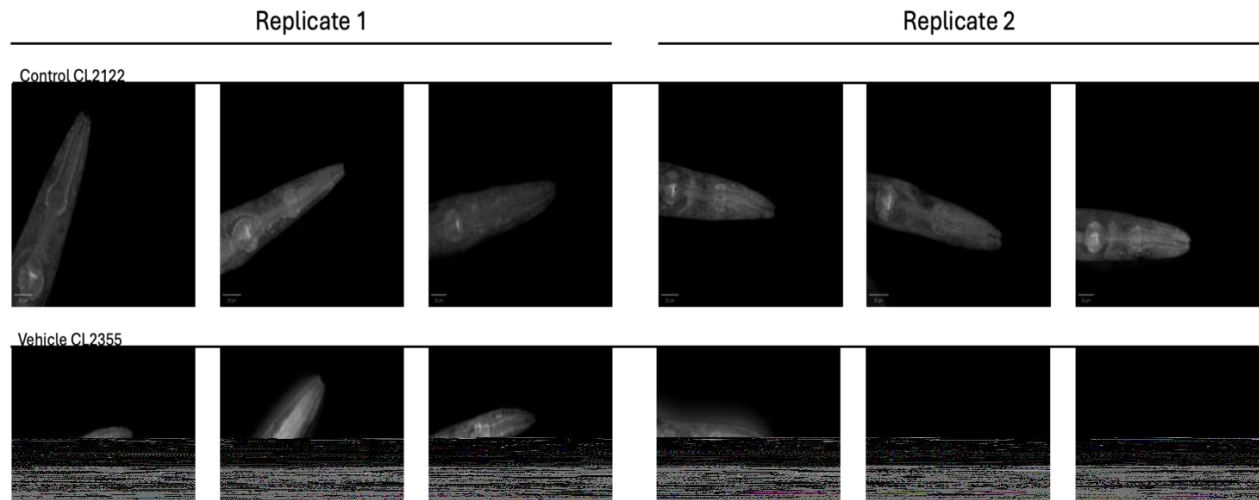


Figure 30. Thioflavin S staining cannot distinguish A β deposits between vehicle-treated, rapamycin-treated CL2355, and CL2122 control animals. Representative fluorescence micrographs of the head region of CL2122 (non-transgenic control; n = 19), vehicle-treated CL2355 (n = 24), and rapamycin treated CL2355 (n = 18) animals stained with 0.125% Thioflavin S (ThS) following freeze-crack fixation. Mean ThS fluorescence intensity was quantified in a standardized rectangular region of interest in the head region using ImageJ. No statistically significant differences in ThS fluorescence were detected between groups in two independent biological replicates, suggesting that ThS staining under the current protocol lacks

sufficient sensitivity to resolve A β deposit burden in the CL2355 pan-neuronal strain. Scale bar, 20 μ m. Visualization at 20 $\times$ magnification with DAPI filter set (excitation $\approx$ 360 nm, emission $\approx$ 460 nm).

3.9 Rapamycin selectively attenuates A β oligomers expression in Transgenic *C. elegans*

CL4176

To investigate whether rapamycin treatment modulates A β protein levels and aggregation in CL4176 worms, western blot analysis was performed using the 6E10 monoclonal antibody. Actin was used as a loading control for normalization across all conditions. Analysis was conducted across two independent biological replicates, each comprising three biological samples per condition (vehicle and rapamycin 20 μ M).

Immunoblotting revealed the presence of four distinct immunoreactive bands at approximately 4, 20, 25, and 60 kDa (Figure 31A). The 4 kDa band, consistent with A β monomer, was not consistently detected across experiments; it was observed in only one of the two independent replicates, indicating that monomeric A β accumulates at levels that are near or below the reliable detection threshold under these experimental conditions. The 20, 25, and 60 kDa bands, which are consistent with low- and high-molecular-weight A β oligomeric assemblies, were detected in both replicates.

Quantification of band intensities, normalized to beta actin, revealed that rapamycin treatment did not significantly alter levels of the 4 kDa monomeric species compared to vehicle-treated controls (Figure 31B; $p > 0.05$). Similarly, no significant difference was observed for the 20 kDa oligomeric species ($p > 0.05$), though a trend toward reduced levels was apparent in rapamycin-treated animals (vehicle: 1.20 ± 0.38 ; rapamycin: 0.43 ± 0.17). In contrast, rapamycin treatment resulted in a

statistically significant reduction in both the 25 kDa ($p < 0.05$) and 60 kDa ($p < 0.05$) A β species relative to vehicle control (Figure 31B).

Collectively, these findings demonstrate that rapamycin selectively attenuates the accumulation of higher-molecular-weight A β oligomeric species in CL4176 worms, while monomeric and lower oligomeric forms appear less consistently affected.

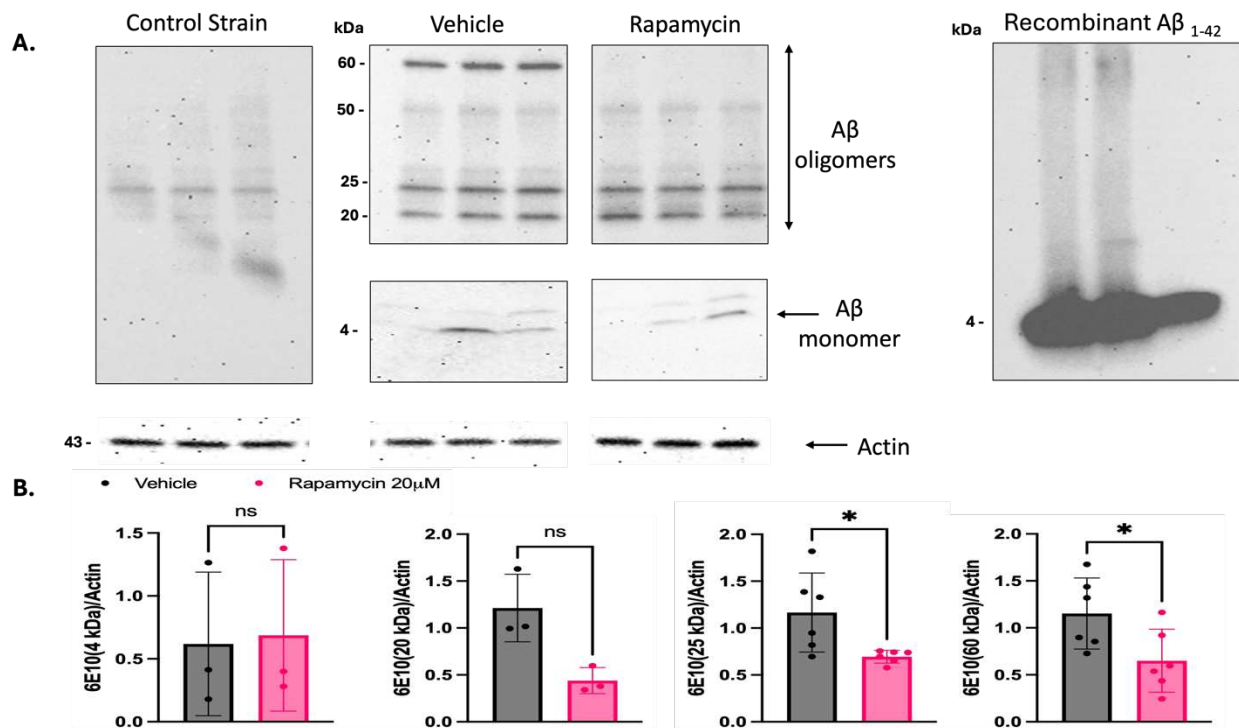


Figure 31. Rapamycin selectively reduces higher-molecular-weight A β oligomeric species in CL4176 transgenic *C. elegans*. (A) Representative Western blots of CL4176 worm lysates from vehicle-treated and 20 μ M rapamycin-treated animals, probed with anti-A β antibody (6E10 for monomeric species; D54D2 for oligomeric species) and anti- β -actin (loading control). Four immunoreactive bands were detected at approximately 4 kDa (monomer), 20 kDa, 25 kDa, and 60 kDa (oligomers). (B) Densitometric quantification of band intensities normalized to β -actin, expressed relative to vehicle control (mean $\pm$ SEM; $n = 2$ biological replicates, 3 biological samples per condition). Rapamycin treatment produced a statistically significant reduction in the 25 kDa ($p < 0.05$) and 60 kDa ($p < 0.05$) bands, while the 4 kDa and 20 kDa bands were not significantly different between groups. Statistical analysis by unpaired Student's t-test in GraphPad Prism.

3.10 Summary of Phenotypic Rescue Across Assays

Across the orthogonal assays presented in this chapter, treatment with 20 μ M rapamycin produced consistent and statistically significant improvements in every A β -relevant readout examined. In the CL4176 paralysis model, rapamycin extended PT₅₀ from 7.7 to 9.5 hours and significantly delayed the entire paralysis curve ($p = 0.0017$). In the CL2355 chemotaxis assay, rapamycin restored the chemotaxis index toward non-transgenic levels (one-way ANOVA $p = 0.015$) without altering behavior in the non-transgenic CL2122 control, confirming A β selective rescue. Kaplan–Meier survival analysis extended lifespan in CL2355 (log-rank, Bonferroni-adjusted $p < 2 \times 10^{-16}$), with the most pronounced effect in the A β -expressing background, and combined survival–locomotion analysis demonstrated preservation of healthspan. At the molecular level, rapamycin selectively reduced high-molecular-weight A β oligomeric species (25 kDa and 60 kDa, $p < 0.05$ each).

Two features of the data set are particularly noteworthy. First, the rescue is strain-selective: where assay design permitted direct comparison, rapamycin acted on the A β -expressing strain without measurably altering the non-transgenic control, supporting engagement with proteotoxic biology rather than non-specific behavioral or physiological stimulation. Second, the rescue is phenotypically broad: improvements of muscle-driven (paralysis, crawling, swimming) and neuron-driven (chemotaxis, lifespan, healthspan) outputs, and short (hours) and long (days) timescales alike. The interpretation and mechanistic implications of these findings are addressed in Chapter 4.

CHAPTER 4: DISCUSSION

4.1 Rapamycin dose optimization

The present preformed a systematic, multi-round dose-optimization to identify a working concentration of rapamycin that produces a reproducible drug-attributable delay in A β -induced paralysis in the CL4176 transgenic *C. elegans* strain. Across four sequential rounds of optimization, 20 μ M was identified as the optimal working concentration and was selected for all subsequent experiments reported in this thesis. The initial broad-range screen revealed that none of the tested concentrations produced a pronounced delay in the paralysis relative to vehicle (1% DMSO). Notably, the two lowest concentrations (1 and 10 μ M) reached statistical significance compared to vehicle. The two higher concentrations (50 and 100 μ M) were statistically non-significant relative to vehicle in this round, which was initially ambiguous but was clarified in the subsequent matched-vehicle experiment. Taken together, these data indicated that the initial dose study failed to identify a clear protective dose of rapamycin: the apparent worsening at low concentrations and the apparent equivalence-to-vehicle at higher concentrations both raised the alternative hypothesis that the observed inter-group differences were being driven by the differing concentrations of DMSO carried into each plate rather than by rapamycin itself. The second round each rapamycin concentration was paired with its corresponding DMSO vehicle, revealed a critical confound: at higher DMSO concentrations (0.5% and 1% v/v), the vehicle itself conferred significant protection against A β -induced paralysis, independent of rapamycin. This finding is consistent with previous reports demonstrating that DMSO at concentrations exceeding 0.5% can alter membrane fluidity and protein aggregation dynamics in *C. elegans* ⁶¹. Consequently, the

apparent rescue observed at 50 and 100 μ M rapamycin could not be attributed to the drug itself, as it was statistically indistinguishable from the matched DMSO vehicle alone. By contrast, the protective signals at 1 and 10 μ M, though modest, were not confounded by a vehicle effect, since DMSO at 0.01% and 0.1% did not differ significantly from untreated controls (plain NGM). A principal limitation of this experiment is its short duration: the assay was designed as a rapid screen to compare rapamycin against its DMSO vehicle within a narrow observation window, rather than as a definitive long-term proteotoxicity study. Guided by these results, follow-up studies focused on rapamycin concentrations below 50 μ M, and the higher concentrations (50 and 100 μ M) were excluded from subsequent investigations, as the substantial vehicle effect at the corresponding DMSO concentrations prevents a definitive interpretation of the pharmacological contribution of rapamycin. To evaluate the effect of the lower-dose treatments, a third, extended paralysis assay was conducted comparing 1 and 10 μ M rapamycin against their matched vehicles. All four conditions produced essentially indistinguishable paralysis trajectories and did not produce a meaningful pharmacological effect. This result effectively eliminated the <10 μ M range from further consideration and redirected the dose-finding effort toward an intermediate range. The fourth and final round of dose-finding focused on 20 and 30 μ M rapamycin, each matched with their respective DMSO vehicles (0.2% and 0.3% v/v), alongside an untreated control. Both concentrations produced clear, biologically meaningful delay in the paralysis. Critically, both rapamycin doses were significantly different from their matched vehicles, formally establishing that the protective effect was pharmacologically driven rather than vehicle-mediated. Furthermore, the two rapamycin doses were statistically equivalent to one another ($p = 1.000$), indicating that the protective effect had plateaued at 20 μ M and that no additional benefit was conferred by increasing the dose to 30 μ M. Taken together, these observations support the selection of 20 μ M

as the working concentration for all subsequent experiments. This choice is based on three reasons: it is the lowest concentration at which a statistically drug-attributable reduction in paralysis was observed; it provides equivalent protection to the higher 30 μ M dose with no measurable additional benefit; and it minimizes both the absolute rapamycin load and the corresponding DMSO vehicle concentration, thereby reducing the risk of vehicle-mediated confounds in the following experiments.

Several caveats associated with this dose-finding work should be noted. The broad-range scan used a single DMSO (1%) concentration as a vehicle rather than matched-to-treatment controls, which contributed to the unexpected results that were subsequently resolved in the matched-vehicle experiment. Additionally, the dose-finding work was conducted exclusively in the temperature-inducible CL4176 strain, and the selected 20 μ M concentration was assumed to be appropriate for the pan-neuronal CL2355 strain and their respective non-transgenic controls.

Finally, It should also be acknowledged that all dose-finding experiments reported in this section were conducted as single biological replicates ($n = 1$), and several assays were intentionally designed as short-duration rapid screens rather than full longitudinal studies. While this approach was appropriate for the repetitive and exploratory nature of dose optimization, it means that the individual experiments lack the statistical power and reproducibility expected of definitive endpoint assays. The p-values reported here should therefore be interpreted as preliminary indicators that guided concentration selection rather than as conclusive evidence of pharmacological efficacy. All subsequent experiments presented in this thesis, performed at the confirmed working concentration of 20 μ M rapamycin.

4.2 Rapamycin Delays Muscle-Specific A β Proteotoxicity: Paralysis and Locomotion

The significant delay in A β -induced paralysis and the broad improvement of crawling and swimming locomotion parameters are the most direct demonstrations that rapamycin alleviates the functional consequences of A β proteotoxicity in muscle tissue. These findings are consistent with earlier reports in mammalian and invertebrate models showing that Rapamycin treatment enhances proteostatic clearance of A β which contribute to the progressive motor failure observed in CL4176 animals after temperature upshift.

The improvements in peristaltic speed, center-point speed, wavelength, and mobility idle time collectively indicate a qualitative restoration of coordinated muscle contraction rather than improvement in a single locomotor parameter. This pattern is particularly informative because the four parameters are mechanistically distinct: peristaltic speed reflects the integrated output of body-wave amplitude and frequency; center-point speed captures net translational displacement; wavelength reports on the spatial frequency of body-wall muscle contractions; and idle time quantifies pausing behavior, which is a sensitive indicator of overall neuromuscular functions in aging nematodes. The fact that all four were improved by rapamycin argues against a simple off-target stimulant effect and is more consistent with a systemic improvement in the proteostatic environment of body-wall muscle. This interpretation is further supported by the locomotion data from the swimming assay, where circuit-specific differences between crawling and swimming (driven by distinct interneuron populations) produced similar improvements across independent readouts, strengthening the conclusion that rapamycin acts on a shared upstream pathogenic process rather than on circuit-specific components.

4.3 Rapamycin Rescues Neuronal A β Toxicity: Chemotaxis and Selectivity

The rescue of the chemotaxis deficit in CL2355 without any measurable effect on the non-transgenic CL2122 control, is one of the most compelling results of this study. Chemotaxis in *C. elegans* is mediated by the AWC and AWA olfactory neurons⁵¹ and deficits in benzaldehyde attraction specifically reflect disruption of neuronal olfactory signaling rather than generalized locomotor impairment. The fact that rapamycin improved CI selectively in the A β -expressing strain, while leaving the baseline chemotaxis of healthy animals intact, strongly argues that the drug is engaging with A β -driven pathology rather than exerting a non-specific pharmacological effect on olfaction or motor performance.

4.4 Lifespan and Healthspan: Disease-Modifying Versus Pro-Longevity Effects

Lifespan and healthspan are related but distinct concepts in aging biology. Lifespan is a purely temporal measure, the chronological duration of survival from birth (or hatching) to death⁶². Healthspan, by contrast, refers to the period of life spent free of significant functional decline, that is, the duration during which an organism retains a defined threshold of physiological and behavioral competence such as coordinated locomotion, sensory function, and reproductive capacity⁶². An intervention can therefore extend lifespan without extending or improving healthspan if it prolongs survival during a period of accumulating frailty. In the other hand, lifespan and healthspan can be experimentally uncoupled, as demonstrated in *C. elegans* longevity mutants that live longer while spending a proportionally greater fraction of that extended life in a state of functional decline⁶². This distinction motivated the combined survival–locomotion analysis performed in the present study, which was designed to determine whether rapamycin extends

chronological survival, functional healthspan, or both, rather than assuming that the two necessarily track with one another.

The pronounced extension of Kaplan–Meier survival in rapamycin-treated CL2355 animals raises the question of whether rapamycin is acting as a disease-modifying agent (by mitigating A β -specific pathology) or as a generic geroprotector (by its well-established pro-longevity effects on the mTOR pathway). The near-identical survival curves of vehicle and rapamycin treated CL2122 animals under the experimental conditions used here suggest that the pro longevity effect of rapamycin was modest in the absence of A β , whereas the survival gain in CL2355 was large and highly significant. This asymmetry supports a disease-modifying interpretation: rapamycin's primary benefit in this context is the attenuation of A β -driven mortality rather than a generic extension of lifespan.

Importantly, the combined survival–locomotion analysis demonstrated that rapamycin improves healthspan rather than simply prolonging survival in an impaired state. The progressive delay in the onset of motor decline in rapamycin-treated CL2355 worms, indicates that the drug maintains neuromuscular integrity over time. In aging research, the distinction between lifespan extension and healthspan extension is of cardinal importance: an intervention that extends life without preserving function is of limited therapeutic value ^{63,64}. The present data demonstrate that rapamycin achieves both in the A β proteotoxic context.

4.5 Molecular Correlates: Selective Reduction of High-Molecular-Weight A β Oligomers

Western blot analysis of A β species revealed that rapamycin selectively reduced the 25 kDa and 60 kDa A β oligomeric bands ($p < 0.05$ each) without significantly affecting the 4 kDa monomer or the 20 kDa lower oligomeric species. These findings are consistent with a growing body of

evidence demonstrating that pharmacological inhibition of the mammalian target of rapamycin complex 1 (mTORC1) does not uniformly suppress all A β species but rather exerts a preferential effect on higher A β aggregation through the autophagy-lysosomal pathway^{38,65,66}. Rapamycin, as discussed in the introduction chapter, is a classical mTORC1 inhibitor that promotes autophagy by relieving mTORC1-mediated phosphorylation of ULK1 and 4EBP1. It was reported by (Spilman et al. 2010) that rapamycin treatment of transgenic mice significantly reduced soluble A β 42 levels and improved cognitive function, with increased autophagy observed in neurons from treated animals. Similarly, (Jiang et al.) showed that temsirolimus, a rapamycin analog, promoted autophagic clearance of A β and provided neuroprotection in both cellular and animal AD models. However, none of these studies distinguished the clearance of specific oligomeric A β species by molecular weight, a gap that our data now begin to address. The large A β oligomers migrating near 60 kDa have been documented by (Barghorn et al.) and described a water-soluble globular A β 42 oligomer termed the ‘globulomer’ found and detected in the brains of both AD patients and A β 42-overproducing transgenic mice. Crucially, the globulomer was formed independently of the fibrillar aggregation pathway and displayed distinct biochemical properties from both monomers and amyloid fibrils. (Lutter et al.) subsequently confirmed and extended these findings and demonstrating that the A β 42 oligomers consistently produced a band at ~60 kDa on Tris-glycine SDS-PAGE gels, as well as 25 and 37 kDa. (Liu et al.) further characterized a class of large A β oligomers demonstrating that the high-molecular-weight A β species represent a biochemically and structurally distinct category from both monomers and fibrils. The preferential reduction of higher-molecular-weight species by rapamycin is therefore consistent with enhanced autophagic clearance of aggregated A β as the primary mechanism of action.

The absence of a significant effect on monomeric A β is equally informative. Because monomeric A β_{1-42} is produced constitutively by the temperature-inducible transgene, its steady-state level reflects the balance between ongoing production (which rapamycin does not affect) and degradation.

The inability of Thioflavin S staining to detect significant differences between transgenic and non-transgenic animals, or between vehicle- and rapamycin-treated CL2355 animals, most likely reflects a detection sensitivity limitation of the ThS assay under the imaging conditions employed. ThS binds with β -sheet amyloid fibrils with high selectivity but low sensitivity for diffuse or non-fibrillar oligomeric deposits ⁶⁷, which are the predominant pathogenic A β species in early-stage neuronal A β models and the very assemblies that are most functionally toxic ⁶⁸.

4.6 Strengths and Limitations

Several methodological strengths should be acknowledged. The use of two genetically distinct *C. elegans* A β models (tissue-specific muscle versus pan-neuronal) allowed the consequences of A β proteotoxicity to be assessed in functionally different contexts and enabled cross-model validation. The systematic, vehicle-matched dose-finding approach rigorously identified a pharmacologically active concentration and explicitly resolved the confounding contribution of DMSO, a limitation that has been underappreciated in some prior *C. elegans* rapamycin studies. The deployment of multiple orthogonal functional readouts (paralysis, crawling, swimming, chemotaxis, lifespan, healthspan) greatly strengthens the evidence for a genuine protective effect, since each assay has distinct technical failure modes and potential confounds.

Several important limitations must also be acknowledged. First, the number of biological replicates varies across assays. The paralysis, locomotion, and dose-finding experiments were performed

with one biological replicate, although within-experiment sample sizes were adequate (≥ 50 worms per condition). The chemotaxis and lifespan assays were performed with two to three biological replicates, providing greater confidence in those results. All conclusions should therefore be interpreted in the context of the replication level of each experiment.

Second, the present study does not directly measure autophagic flux, mTORC1 activity, or lysosomal function in rapamycin-treated worms. While the phenotypic and molecular data are consistent with autophagy-mediated clearance of A β oligomers as the mechanism of protection, this remains an inference rather than a demonstrated mechanistic link. These experiments are planned as part of the future directions described in Chapter 5.

Third, the dose-finding work was conducted exclusively in the CL4176 strain and the selected 20 μ M concentration was assumed to be appropriate for CL2355 and CL2122. While the consistent biological responses observed in CL2355 across multiple assays suggest that this assumption was reasonable, formal dose–response experiments in the neuronal model were not performed.

Fourth, *C. elegans* lack a blood-brain barrier, a mammalian-scale immune system, and the full complement of glial cell types present in the vertebrate nervous system, all of which modulate drug pharmacokinetics and A β pathology in humans. While the worm conservation of fundamental proteostatic pathways provides mechanistic validity, the translational relevance of specific effect sizes to human Alzheimer's disease requires validation in higher organisms. Fifth, this study administered rapamycin as a single dose rather than as multiple discrete doses and did not incorporate pharmacokinetic sampling to determine the internal drug concentrations achieved in worm tissue over time. Future studies incorporating multiple dosing regimens, as outlined in Chapter 5, will be required to determine the minimal effective and optimal dosing interval. Sixth, the actual internal concentration of rapamycin achieved within worm tissue over the course of

exposure was not measured. The 20 μ M value used throughout this study refers to the nominal concentration of rapamycin in the culture medium, not a verified internal dose so without direct quantification, it cannot be assumed that this external concentration translates linearly, or even reliably, into a defined internal tissue concentration discussed in Section 1.7. Confirming the internal exposure achieved would require targeted mass spectrometry (liquid chromatography–tandem mass spectrometry, LC–MS/MS) quantification of rapamycin in whole-worm lysates across the treatment period, an analysis that was beyond the scope of the present study but is recommended as a priority for future work.

4.7 Relationship to Existing Literature

The findings of the present study are broadly consistent with, and extend, prior work on rapamycin in *C. elegans* and mammalian AD models. (Robida-Stubbs et al.) demonstrated that dietary rapamycin extends *C. elegans* lifespan in a TFEB/HLH-30-dependent manner and reduces A β aggregation in a pan-tissue model, suggesting that transcriptional upregulation of the lysosomal-autophagic machinery contributes to rapamycin's pro-longevity effects. The present data extend this observation by demonstrating that 20 μ M rapamycin specifically rescues behavioral deficits in tissue-targeted A β models and selectively reduces higher-molecular-weight A β oligomeric species at the protein level. In transgenic rodent models of AD, Spilman et al. (2010) and Caccamo et al. (2010) reported that rapamycin reduces amyloid plaque burden, decreases A β_{1-42} levels, and improves cognitive performance in 3 $\times$ Tg-AD and APP/PS1. The selective reduction of higher-order A β oligomers observed in the present study is consistent with autophagy-mediated clearance of proteotoxic aggregates as the primary mechanism, as proposed in the mammalian literature.

The strain-selective rescue of chemotaxis (preserved in CL2122 but significantly improved in CL2355) parallels the selectivity of rapamycin's cognitive effects in rodent models, where cognitive improvements are observed in AD transgenic but not wild-type mice. This parallel suggests that *C. elegans* CL2355 captures a functionally analogous form of A β -specific neural circuit dysfunction, lending further support to its use as a platform for evaluating candidate AD therapeutics.

CHAPTER 5: CONCLUSION AND FUTURE DIRECTIONS

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5.1 Principal Conclusions

This thesis provides the first systematic, vehicle-controlled characterization of rapamycin's neuroprotective effects in *Caenorhabditis elegans* models of Alzheimer's disease-associated amyloid-beta proteotoxicity. The study addressed a specific gap in the literature: while rapamycin has been shown to extend lifespan and reduce A β burden in mammalian transgenic AD models, its ability to rescue A β -specific phenotypes in isolated muscle and neuronal expression systems and the dose requirements and molecular correlates of that rescue in *C. elegans* had not been characterized.

Across an integrated panel of functional, behavioral, longevity, and biochemical assays, 20 μ M rapamycin produced the following principal outcomes:

- **Delayed A β -induced paralysis.** Rapamycin extended the PT50 of A β -induced paralysis in CL4176 animals by approximately 1.8 hours (log-rank $p = 0.0017$), demonstrating significant protection against muscle-specific A β proteotoxicity.
- **Improved neuromuscular function.** Rapamycin broadly enhanced crawling locomotion (peristaltic speed, center-point speed, wavelength, and mobility idle time) and swimming locomotion (wave initiation rate, swimming speed, dynamic amplitude, and wave attenuation) in CL4176 animals, providing circuit-independent evidence for restoration of coordinated neuromuscular performance.

- **Rescued neuronal behavioral deficit.** Rapamycin restored the chemotaxis index in CL2355 pan-neuronal A β animals toward non-transgenic levels ($p = 0.015$), without altering behavior in the non-transgenic CL2122 control and establishing strain-selective, A β -directed efficacy.
- **Extended lifespan and healthspan.** Rapamycin significantly extended Kaplan–Meier survival in CL2355 animals (log-rank $p < 2 \times 10^{-16}$), with the most pronounced benefit in the A β -expressing animals. Combined survival–locomotion analysis demonstrated that rapamycin preserved neuromuscular function over time, extending healthspan alongside chronological lifespan.
- **Selectively reduced higher-molecular-weight A β oligomers.** Western blot analysis revealed a significant, selective reduction of 25 kDa and 60 kDa A β oligomeric assemblies in rapamycin-treated CL4176 animals ($p < 0.05$), without a significant effect on monomeric or lower oligomeric A β species biochemical evidence consistent with autophagy-mediated clearance of proteotoxic oligomeric aggregates.

Overall, these findings establish that rapamycin confers consistent, broad-spectrum, and A β -selective neuroprotection in *C. elegans* models of Alzheimer's disease.

5.2 Contribution to the Field

This work contributes to the field in several ways. First, it provides the most comprehensive phenotypic characterization to date of rapamycin's effects in *C. elegans* A β models, spanning from acute (paralysis, locomotion), subacute (chemotaxis), and chronic (lifespan, healthspan) endpoints. Second, the rigorous, vehicle-matched dose-finding strategy resolves an underappreciated methodological confound in *C. elegans* pharmacological studies and establishes a template for future work using DMSO-solubilized compounds. Third, the combined survival–locomotion framework demonstrates that rapamycin extends healthspan, not merely lifespan, which is the more therapeutically meaningful outcome in the context of a dementia-causing disease.

5.3 Future Directions

Several lines of investigation are prioritized for future work arising from this thesis. The most immediate priority is the mechanistic validation of the mTOR–autophagy axis as the causal driver of rapamycin's neuroprotective effects.

A second major future direction concerns the evaluation of tau pathology. The present study focused exclusively on A β proteotoxicity; however, the interaction between A β and tau pathology is central to the amyloid cascade hypothesis and to the full spectrum of AD neuropathology. *C. elegans* strains expressing human tau offer tractable models for examining whether rapamycin can attenuate tau-associated motor deficits and whether the A β and tau rescue phenotypes are additive or synergistic. These experiments would broaden the therapeutic relevance of the present findings and are prioritized for subsequent studies.

A further priority is the incorporation of multiple, interval defined dosing regimens rather than the single concentration used in this study. Rapamycin was maintained at a constant nominal concentration in the culture medium for the duration of each assay which is an exposure profile

that does not capture the peak-and-trough pharmacokinetic, extended elimination half-life, and saturable absorption that characterize rapamycin dosing in mammals ^{26,27}. Future work should compare repeated intermittent exposure against the continuous-exposure used here, both to determine whether the same magnitude of protection against A β proteotoxicity can be achieved with lower cumulative drug exposure and to establish whether intermittent dosing mitigates any chronic off-target consequences of sustained mTORC1 inhibition.

Finally, the evaluation of combinational treatment with CBD and Trazadone with the goal of studying AD pathology with different mechanism of protection.

5.4 Final Remarks

Alzheimer's disease remains one of the most devastating and scientifically challenging conditions of our time, and the accelerating prevalence of dementia in an aging global population makes the need for disease-modifying therapies increasingly urgent. The traditional drug development pipeline (from target identification and de novo compound synthesis through phase III clinical trials) routinely spans fifteen to twenty years and costs more than millions of dollars per approved agent. Drug repurposing offers a fundamentally different and strategically compelling approach: by reorienting compounds with established safety profiles, known pharmacokinetics, and existing regulatory approval toward new therapeutic indications, the repurposing framework compresses timelines, reduces costs, and substantially de-risks early-phase clinical investigation. Rapamycin exemplifies the promise of this strategy. As an FDA-approved macrolide with decades of clinical use in clinics, its safety, tolerability, and pharmacokinetic behavior in humans are extensively characterized which is a critical advantage over investigational compounds entering the AD pipeline without this foundation.

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