Investigating the Effects of Amyloid Plaques on Oxidative Stress throughout the Life Cycle of *Caenorhabditis elegans*

Grant Proposal

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Abstract

The prevalence of Alzheimer's Disease (AD) is increasing, along with the average age of the population. There is no cure for AD due to a lack of knowledge on the underlying pathology of the disease. Amyloid plaques and oxidative stress are especially puzzling because there is conflicting evidence on which one appears first, and they both cause an increase in the amount of the other. Both Amyloid plaques and oxidative stress are found in both healthy and Alzheimer's patients, which allows this research to also apply to the processes of general aging. In order to learn more about the mechanisms associated with the cognitive decline of Alzheimer's patients, we will use *Caenorhabditis elegans* to test the effects of aging on the amounts of Amyloid plaque-induced oxidative stress. A novel strain of transgenic *C. elegans*, which continuously expresses GFP as a reporter for oxidative stress and expresses Amyloid beta when warmed will be used. We will induce Amyloid plaques at different stages in the life cycle of the worms, then the worms will be dyed with Congo red to ensure the expression of amyloid plaques. We hope to find that as the age of the *C. elegans* increases, the levels of ROS produced in response to amyloid plaques will also increase. This research will give us further insight on the pathology of Amyloid plaques and oxidative stress in Alzheimer's, as well as in the process of aging. This relationship helps to explain the age-dependency and pathology of Alzheimer's.

Keywords: Alzheimer's, Amyloid plaques, oxidative stress, Reactive Oxygen Species, aging

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According to an estimate by the World Health Organization, the number of people who will suffer from dementia in 2030 is 78 million (Zhang et al., 2024). Alzheimer's disease (AD) is currently the most common form of dementia. AD is a neurodegenerative disease that is most often found in older adults, especially in people above the age of 60, meaning that as the average life expectancy increases, so does the incidence of Alzheimer's (Zhang et al., 2024). Common symptoms of AD are memory loss, personality changes, difficulty with thinking, and dysfunctions in the motor skills and executive functions of the patient.

Patients with Alzheimer's are known to have large accumulations of amyloid-β (Aβ) plaques, neurofibrillary tangles (NFTs), neuroinflammation, synaptic dysfunction, and mitochondrial and bioenergetic disturbances (Zhang et al., 2024). There are various hypotheses explaining the mechanisms of Alzheimer's, yet no hypothesis can explain every aspect of Alzheimer's disease pathology. Due to the current lack of understanding of the underlying pathology, most cases of Alzheimer's are diagnosed at late stages, which lowers the chance that treatments will work. The treatments are also less effective because they can only treat the effects of the underlying pathology, which will never cure the disease and has limited effectiveness. The current explanation is that a number of factors, such as Aβ, Tau proteins, neuroinflammation, oxidative stress, biometal dyshomeostasis, glutamate imbalance, gut microbiome abnormalities, cholesterol homeostasis disruption, mitochondrial dysfunction, genetic factors, and autophagy abnormalities all combine to cause Alzheimer's (Zhang et al., 2024). Amyloid plaques and oxidative stress are two mechanisms that are highly connected. There has been much debate over which mechanism initially causes the other. It has been difficult to determine the root cause because Amyloid plaques cause Oxidative Stress and Oxidative Stress causes more Amyloid plaques (Chen et al., 2017).

Amyloid Plaques are a hallmark of AD pathology and many of the genetic causes of AD have some relation to the overexpression of Amyloid beta (Bellenguez et al., 2022). Amyloid beta is a protein produced from the Amyloid Precursor Protein as it is broken down by beta- and gamma- secretases. Amyloid plaques are formed when many Amyloid beta proteins combine to form fibrils, which further combine into the plaques

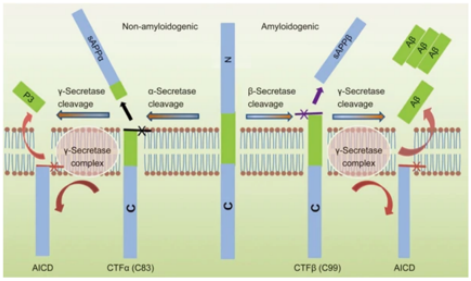


Figure 1. The pathway for the breakdown of Amyloid Precursor Protein (APP) and how Amyloid beta is created (Chen et al., 2017).

In previous research, Caenorhabditis elegans had been used as a model organism to study the pathology of Alzheimer's disease. These worms are transparent, which makes them easy to use for fluorescence imaging, most commonly with GFP (Hutter, 2012). While *C. elegans* cannot generate Amyloid beta peptides naturally, transgenic lines have been created to express the human versions of Amyloid beta (Alvarez et al., 2022) through the mutation of genes known to help produce Aβ associated with Alzheimer's, such as the Amyloid Precursor Protein (Bellenguez et al., 2022). Current research on the mechanisms of AD ties mitochondrial dysfunction and oxidative stress to Amyloid beta, showing that Amyloid beta can cause oxidative stress, damage to mitochondrial DNA, and excess Reactive Oxygen Species (ROS). Reactive Oxygen Species are naturally produced during Oxidative Phosphorylation, but an excess of ROS is damaging to the cell and can contribute to cell death. The oxidative stress and ROS produced by the mitochondrial dysfunction can influence the pathology of Amyloid beta (Gao & Ma, 2022).

Amyloid Plaques and oxidative stress are both found naturally throughout the process of aging. Figure 2 shows the accumulation of Amyloid beta over time in individuals who are developing Alzheimer's (Jagust & Landau, 2021). The levels of Amyloid plaques increase

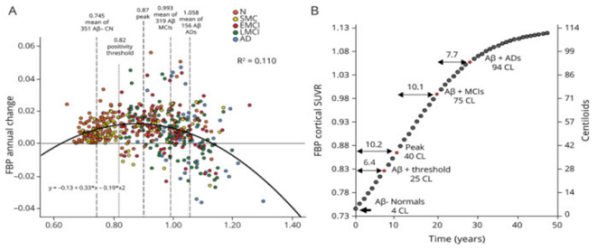


Figure 2. These graphs show the accumulation of Amyloid beta in the brain over time. We can see that while amyloid beta does not always increase, it is elevated in all older patients, although the amount of amyloid beta differs with their cognitive state. CL = Centiloids; CN, N = cognitively normal; EMCI = early mild cognitive impairment; LMCI = late mild cognitive impairment; MCI = mild cognitive impairment; SMC = subjective memory complaints; SUVR =standardized uptake value ratio (Jagust & Landau, 2021).

over time in both individuals who are healthy and those with AD. High levels of amyloid plaque pathology do not always indicate Alzheimer's, which shows that the overexpression of Amyloid beta is related to age. Some patients with high levels of amyloid beta do not actually have the disease or its symptoms. This provides evidence these two mechanisms do not work alone (Zhang et al., 2024). However, the scope of this study is not wide enough to consider the other factors that play into the pathology of Alzheimer's.

There is a lack of knowledge on the interactions between the different mechanisms of AD. Understanding the interactions and relationships between the Amyloid plaques and oxidative stress in AD will help to uncover the underlying mechanism, which in turn will help with prevention, early detection, and more effective treatment of Alzheimer's. As Amyloid plaques and oxidative stress are found in both healthy and Alzheimer's patients, research on the relationship between these two mechanisms throughout the process of aging can have implications for the differentiation of patients with Alzheimer's and healthy patients with high levels of amyloid plaques and oxidative stress that would usually indicate AD. The goal of this project is to understand the difference in the impact of Aβ on oxidative stress based on the age of the *C. elegans*. The work proposed would use the offspring of the transgenic CL2355 strain that overexpress human amyloid beta in pan-neuronal cells when exposed to heat and the CL691 strain that expresses Green Fluorescence Protein (GFP) in response to oxidative stress. These worms will be obtained from the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA) and maintained with the standard agar and E. coli (Kittimongkolsuk et al., 2021). The worms would be exposed to heat at different stages of their life cycles, causing them to produce amyloid plaques. Then, the amount of ROS and Amyloid plaques produced would be monitored using Congo red stain and the expressed GFP. This would be done with the use of a stereo microscope, LEDs, and filters. We expect that as the age of the *C. elegans* increases, the levels of ROS produced in response to amyloid plaques will also increase.

Section II: Specific Aims

This proposal's objective is to determine the impact of age on the effects of Amyloid Plaques on the production of Reactive Oxygen Species. Our long-term goal is to improve the understanding of the effects of age on Amyloid plaque-induced oxidative stress to help the understanding of the age-related pathology of Alzheimer's and potentially lead to better treatment options. The central hypothesis of this proposal is that as the age of the C. elegans at the inducement of Amyloid plaques increases, the amount of ROS produced in response will also increase. The rationale is that both Amyloid plaques and ROS are found in the elderly population, sometimes at levels that would indicate AD even though they have no symptoms of the disease. As part of both the natural aging process and the pathology of Alzheimer's, Amyloid plaques and oxidative stress are at an interesting crossroads between healthy aging and disease pathology. The work we propose here will use C. elegans as a model organism to help clarify the impacts of age on the mechanisms of Amyloid beta-induced oxidative stress.

Specific Aim 1: To determine the impact of Amyloid beta on oxidative stress.

Specific Aim 2: To determine the effect of increases in age on the impact of Amyloid beta on oxidative stress.

The expected outcome of this work is that Amyloid plaques will cause oxidative stress, and that age will affect this process. We expect that as age increases, the impact of amyloid plaques on Oxidative stress will also increase.

Section III: Project Goals and Methodology

Relevance/Significance

The prevalence of Alzheimer's is increasing with the average age of the population, which increases the need for research on both aging and Alzheimer's. This project will help to improve the understanding of Alzheimer's pathology, as well as the processes of aging in the brain by gaining an understanding of how age impacts the pathology of AD.

Innovation

The work proposed is a novel combination of previously used methods, some of which have been modified to research the specific areas of this work. We will use methods of maintaining the *C. elegans* at constant temperatures that are modified versions of heat shock experiments in *C. elegans* (Zevian & Yanowitz, 2014). This work will also use previously used methods of fluorescence marking and detection (Korovesis et al., 2023). This experiment utilizes a novel strain of *C. elegans* to produce Amyloid plaques and express GFP in the presence of Reactive Oxygen Species. This work combines all of these methods to study the effects of age on amyloid beta-induced oxidative stress.

The methods for maintaining the C. elegans were created as a modified version of the general methodology for heart shock experiments in C. elegans. In the heat shock experiments, the worms were heated in an incubator that contained heat-retaining objects to increase the surface area and retain heat to reduce the time taken to bring the incubator back up to temperature after it opened (Zevian & Yanowitz, 2014). While the heat shock methodology was trying to induce heat shock, this project simply proposes inducing amyloid plaques. The temperature of the incubator will be lowered to 16°C to keep these *C. elegans* from expressing Amyloid beta at the wrong time. This will prevent the *C. elegans* from getting heat shock-induced oxidative stress or having other complications that could interfere with the experiment (Zevian & Yanowitz, 2014).

This project required GFP to indicate ROS, as well as worms that could be induced with Amyloid plaques. There are currently no strains that contain both the genes required to induce Amyloid plaques and the genes to express GFP in response to oxidative stress. Our lab does not have the ability to microinject any plasmids into the gonads of the *C. elegans*, therefore we chose to crossbreed the CL2355 and CL691 strains to create a novel strain that contains all necessary genes for this experiment.

The innovation in this project is that we will be inducing the Amyloid beta at different stages in the life cycle to research the effects of age on oxidative stress caused by Amyloid beta. This approach has the potential to help us understand the differences between normal aging and Alzheimer's disease, using a combination of many preexisting methods.

Methodology

Crossbreeding CL2355 and CL691:

Place CL2355 and CL691 *C. elegans* on a plate of Nematode Growth Medium and OP50 *E. coli*. Maintain these plates at 16°C for three days to allow ample time for reproduction. After three days, heat the worms at 35° for 30 minutes to induce Amyloid plaques and oxidative stress. Next, we will wash the worms in a 0.1% solution of Congo red in M9 buffer. Allow the worms to soak in the Congo red solution for 10 minutes. After this, pipette them onto a seeded plate and observe the worms under a stereo microscope with an LED set to 395nm wavelength with a green filter for GFP and an LED set to a 614 nm wavelength with a red filter for Congo red. After observing them, we will return the control to the cooling chamber set to 16°C.

Specific Aim #1:

Determine the impact of Amyloid beta on oxidative stress. The objective is to gain more evidence to help better understand the cycle of amyloid beta and oxidative stress pathology in Alzheimer's disease. We aim to aid in clearing up the debate over whether Amyloid beta causes oxidative stress or not. Our methodology is to synchronize the crossbred worms and each of the four uniformly seeded plates of NGM kept at 16°C. Maintain the four plates at 16°C and allow all worms to hatch, which should take around 14 hours. Once the synchronized worms enter the L2 stage, we will take test group 1 and heat them to 23°C. After 30 minutes, we will wash both the control group and test group with a 0.1% solution of Congo Red in M9. Then, observe the worms under a stereo microscope with an LED set to 395nm

wavelength with a green filter for GFP and an LED set to a 614 nm wavelength with a red filter for Congo red. After observing them, we will return the control to the cooling chamber set to 16°C. Approximately 24 hours later, when the worms are in the L4 stage, we will take test group 2 and move them to the incubator at 23°C. After 30 minutes, wash both the control group and test group with a 0.1% solution of Congo Red in M9. Then, we will observe the worms under a stereo microscope with an LED set to 395nm wavelength with a green filter for GFP and an LED set to a 614 nm wavelength with a red filter for Congo red. After observation and data collection, we would return the control to the cooling chamber set to 16°C. About nine hours later, when the *C. elegans* are in the adult stage, we will take test group 3 and heat them to 23°C. After 2 hours, we would wash both the control group and test group with a 0.1% solution of Congo Red in M9. Finally, stereo microscope with an LED set to 395nm wavelength with a green filter for GFP and an LED set to a 614 nm wavelength with a red filter for Congo red. Our rationale for this approach is that the CL2355 strain must be kept at 16°C to keep them from expressing Amyloid beta before we induce it. GFP is used because of its ease of use and availability. We use four groups of a novel strain of *C. elegans*, three of which will be induced with Amyloid plaques at different times to gather data on the levels of ROS after the induction of Amyloid plaques, and the last group will be the control. The control will not be given Amyloid beta at all.

Justification and Feasibility. The use of a stereo microscope with LEDs and filters is relevant to this project because it is efficient at detecting GFP fluorescence. Figure 3 shows the ability of a camera to detect green colors and an example of GFP fluorescence imaging using LEDs and filters. The efficiency of the camera at detecting GFP with a stereo microscope, LEDs, and filters provides evidence that this methodology will allow us to detect GFP in *C. elegans*.

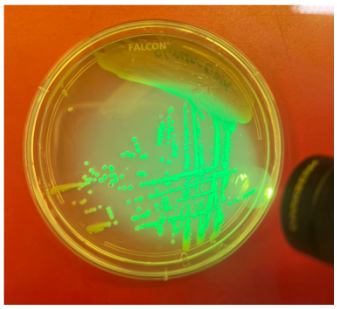


Figure 3. A 395 nm wavelength LED angled at 45° towards the GFP-expressing *E. coli* viewed through a yellow filter.

Summary of Preliminary Data. We found that a camera was able to detect the GFP-expressing bacteria, which shows that the detection of fluorescence using LEDs and filters with a stereo microscope is feasible.

Expected Outcomes. The overall outcome of this aim is that Amyloid plaques cause excess ROS. This knowledge will be used to gain a better understanding of aging and Alzheimer's.

Potential Pitfalls and Alternative Strategies. We would like to use a fluorescence microscope. This would make the data more precise and allow us to determine where the fluorescence is localized.

Specific Aim #2:

Determine the effect of increases in age on the impact of Amyloid beta on oxidative stress. The objective is to find the differences, if there are any, in the amount of Amyloid plaque-induced oxidative stress as the *C. elegans* age. Our approach is included above in Specific Aim #1. Our rationale for this approach is that comparing the amounts of Amyloid plaques at the different stages of the life cycle will allow us to determine the differences in the effects of amyloid beta on oxidative stress and ROS production as the *C. elegans* age. We will compare the amounts of ROS produced from when the *C. elegans* are first induced with the amyloid plaques with the control group at each stage of the life cycle.

Justification and Feasibility. The use of the offspring of the CL2355 and CL691 strains is feasible for this project. In figure 4, the worm shows both GFP and Congo red, indicating that it contains genes from both strains. This worm was heated according to the methodology for crossbreeding above. The nematode expressed GFP, meaning that it contained the genes for expressing GFP in response to oxidative stress form the CL691 strain. Congo red was visible in the *C. elegans*, showing that it had produced Amyloid plaques when heated due to genes from the

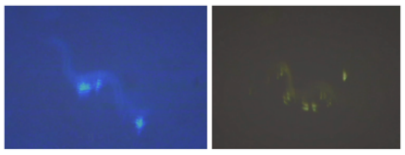


Figure 4. Both images show the same *C. elegans*. The image on the left shows the worm expressing GFP, while the image on the right shows the worm stained with Congo red.

CL2355 strain. The appearance of both types of fluorescence in the same worm provides evidence for the ability of these strains to crossbreed.

Summary of Preliminary Data. We found that the CL691 and CL2355 strains were able to crossbreed. This provides evidence that the worms are crossbred, and that the project is feasible.

Expected Outcomes. The overall outcome of this aim is that the amount of ROS produced in response to Amyloid plaques will increase with age. This knowledge will be used to gain a better understanding of aging, as well as the role aging plays in the pathology of Alzheimer's.

Potential Pitfalls and Alternative Strategies. We expect that these results would be more accurate with the inclusion of other mechanisms found in Alzheimer's. We propose the use of human neuronal cells to better simulate the actual conditions of AD. As Alzheimer's is a purely human disease, it is challenging to model all of its mechanisms in another organism. This method would require much stricter protocols and would have many more ethical considerations, which is why it will not be used for this experiment.

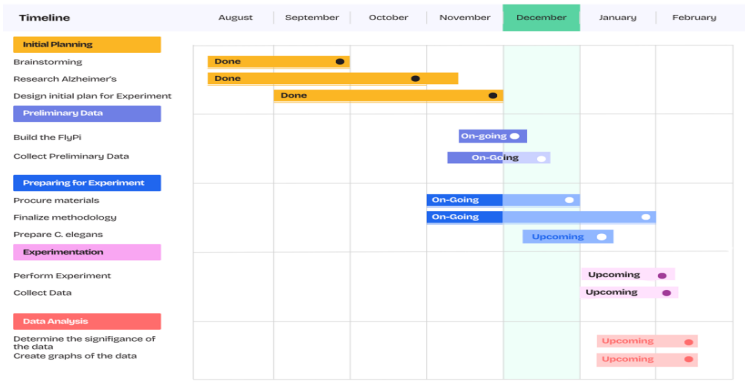
Section III: Resources/Equipment

We will use an incubator, cooling chamber, CL2355 and CL691 *C. elegans*, Congo red, M9 buffer, pipettes, OP50 *E. coli*, Nematode Growth Medium, LEDs, light filters, a microscope camera, and a stereo microscope.

Section V: Ethical Considerations

We will treat the C. elegans ethically. To do this we will keep them fed and ensure that they are kept in the proper environment. We will be inducing Amyloid Plaques in them, which will harm them. However, we will try to keep them comfortable in every other way.

Section VI: Timeline



Section VII: Appendix

Purpose of the funding opportunity Novel Mechanism Research on Neuropsychiatric Symptoms (NPS) in Alzheimer's Dementia: The goal of this Notice of Funding Opportunity is to encourage applications for studies that will enhance knowledge of mechanisms associated with neuropsychiatric symptoms in persons with Alzheimer's disease or Alzheimer's disease-related dementias. The findings are expected to advance mechanistic understanding of both biobehavioral and neurobiological pathways leading to NPS.

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