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Investigating the Role of Amyloid Beta Peptides in Inflammation and Alzheimer's Disease Pathogenesis

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INVESTIGATING THE ROLE OF AMYLOID BETA PEPTIDES IN INFLAMMATION

AND ALZHEIMER'S DISEASE PATHOGENESIS

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48 Pages

Alzheimer's Disease (AD) is a neurodegenerative disease characterized by severe memory decline and cognitive impairments. In AD patients' brains, aggregates of amyloid beta (Aβ) peptides, called amyloid plaques, are hypothesized to trigger innate immune responses that contribute to AD pathogenesis. Interestingly, recent studies demonstrated that an increased level of Aβ protected the host against pathogen infections. Using *Drosophila* as a model organism, our preliminary data showed that the loss of the *Drosophila* APP homolog, *APPL*, led to immune deficits against parasite infection, and that overexpression of *Drosophila* Aβ produced an inflammatory phenotype. These findings suggested that Aβ might be required for a successful immune response. Additionally, we produced flies that develop different levels of Aβ aggregation and examine the inflammation responses of these flies during a pathogen infection. We also optimized fly cognition assay to examine the cognitive functions of the Aβ-expressing flies. Our data suggested that there was a correlation between Aβ aggregation and inflammatory responses, and that Aβ-mediated inflammation was associated with cognitive defects. This study helped us to gain a deeper insight on how innate immunity and infection contribute to the development of AD.

KEYWORDS: Amyloid Beta Protein, Alzheimer's Disease, *Drosophila*, Inflammation, Innate Immunity

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If I have seen further, it is by standing on the shoulders of Giants - Isaac Newton

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CHAPTER I: ALZHEIMER'S DISEASE AND AMYLOID BETA

Alzheimer's Disease is a Neurodegenerative Disease

Alzheimer's disease (AD), affecting approximately 50 million people worldwide, is the most common form of dementia (*1*). It is a disease that severely damages cognitive function, inhibiting people from living an independent life. AD is characterized by difficulties with memory, progressive cognitive impairment, dysfunctions in daily activities and abnormal mental and behavioral changes (*2*). AD-mediated molecular changes to the brain begin many years earlier, up to two decades before symptoms appear (*3*).

One of prominent features of AD is the loss of neurons, called cerebral atrophy, which proceeds at a rate of 1% to 4% per year, compared to 0.3% to 0.7% per year in cognitively normal elderly people (*4*). This neuronal loss begins in the entorhinal cortex, a brain region connected to the hippocampus, which is in charge of learning and memory (*5*). The degeneration of these brain areas explains the symptoms of forgetfulness and other cognitive impairments in early-staged AD patients, such as incapability to concentrate and solve problems (*5*). After two to four years, the severe phase of AD was characterized by enlargement of ventricles and omnipresent brain atrophy in the cerebral cortex that controls language, reasoning, sensory processing and conscious thoughts (*5*). Patients become to show more pronounced problems, such as delusions, anger outbursts, and incapability to recognize family members and accomplish independent routine tasks (*5*).

Aβ is Associated with Alzheimer's Disease

At the molecular level, the extracellular buildup of aggregated amyloid beta (Aβ) peptides, called amyloid plaques, is a characteristic of Alzheimer's disease. It is highly conserved across vertebrates; it is at least 400 million years old and found in 60%–70% of vertebrate species (*6*). In normal physiology, Aβ is a small protein (about 4kD), composed of 37–49 amino acid residues and present in brain interstitial fluid, cerebrospinal fluid, plasma, saliva and most tissues in the

body (*7, 8*). The protein is produced from the cleavage of full-length amyloid precursor protein (APP) by the β-secretase and γ-secretase enzymes. Cleavage of APP by γ-secretase might result in different lengths of peptides, leading to size variants, such as Aβ40 and Aβ42. Following this cleavage, Aβ40 and Aβ42 variants either exist as a monomer or accumulate into oligomers, protofibrils, amyloid fibrils and amyloid plaques (*9*), which are illustrated in **Figure 1**.
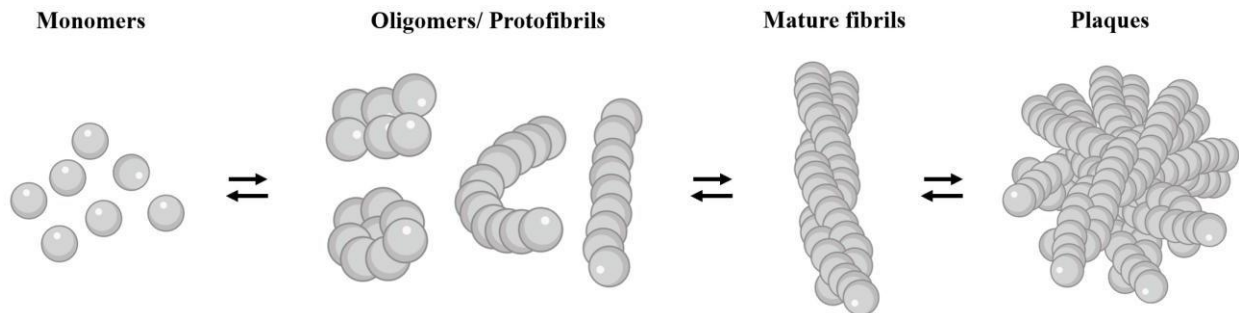
Figure 1: Aβ monomers form oligomers, fibrils, and plaques

Although it remains inconclusive about what structures and variants of Aβ are detrimental or helpful during the development of AD as described in the next chapter, the amyloid plaque structure formed by the Aβ42 variant has been observed in all AD patients and suggested to be the most cytotoxic version. Aβ42 has a higher tendency to aggregate and form amyloid plaques as compared to Aβ40, a more common peptide yet not pathologically associated with AD (*7*). Several point mutations in the amino acid sequence of Aβ42 have been suggested to result in different levels of aggregation and pathological effects in AD patients (*10, 11*). For example, the Arctic mutation, Aβ42[E22G] (Aβ42 with the glutamic acid (E) at position 22 mutated to a glycine (G)) can form aggregates to a far greater extent than wild-type Aβ and displays stronger resistance to protein clearance (*12*). In our study, we aimed to examine five different Aβ variants (**Table 1**) to get a better insight into the different pathological effects of these molecules and how they can be used in therapeutic treatments of Alzheimer's disease.

CHAPTER II: INNATE IMMUNITY CONTRIBUTES TO ALZHEIMER'S DISEASE

Aβ Plays a Protective Role in the Immune System

While Aβ has been characterized in normally aged people (*13*), intriguingly, a growing number of studies revealed a protective role of Aβ in the immune system, a network of cells and organs that defends against foreign molecules and fights infections. Aβ has been recognized as an antimicrobial peptide (AMP) for its ability to bind to bacterial surfaces and agglutinate bacteria and fungus (*11*, *15*). One study also supported this by showing that Aβ mediates pathogen entrapment and agglutination, which increased host survival time against bacterial and fungal infections (*15*). Furthermore, Aβ was suggested to bind to the microbial surface and hinder the growth of at least eight clinically important pathogens, including those commonly causing brain infections (*16*). These findings strongly supported that Aβ deposition could be a protective innate immune response against infection.

Pathogen Infection is Associated with AD

Consistent with research about the role of Aβ in the immune response, it has been suggested that pathogen infection is a risk factor for Alzheimer's disease. Viruses and other microbes can be identified in the brain of most elderly people. Normally, these pathogens remain in a harmless dormant state (*17*). However, AD pathology is correlated with the unusual activation of those pathogens, identified as brain infections caused by bacteria, such as *P. gingivalis* and *C. pneumoniae*, and viruses, including herpes simplex virus 1 (*18–20*). In particular, *P. gingivalis* infection in mice was observed to exacerbate the production of Aβ42 (*20*). Another study showed that 90% of the amyloid plaques in AD patients with herpes infection contained herpes viral DNA (*21*), suggesting the involvement of Aβ during an infection.

Inflammation and Aβ Abundance are Risk Factors for AD

As a first line of defense against pathogens, inflammation occurs when immune cells accumulate at sites of injury or infection (*22*). At the area of inflammation, activated microglia, the main immune cells and phagocytes of the central nervous system, express increased levels of antigens, release inflammatory cytokines and recruit other immune cells (*23*). In AD brains, these brain immune cells can recognize Aβ via specialized pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), complement factors, and scavenger receptors (SRs) (*13*). Once activated by a stimulus, such as an Aβ burden or pathogenic infection, microglia secrete various pro-inflammatory cytokines and chemokines that recruit more microglia and astrocytes to inflammatory sites (*24*). As a pathogen infection progress, prolonged inflammation could be the leading risk factor for the development of AD as upregulated release of proinflammatory cytokines and chemokines has been widely observed in AD brains (*25, 26*).

In addition to the persistent immune responses, clearance of Aβ plaques by microglia or other mechanisms might be impaired or not sufficient to overcome an accumulation of Aβ (*27, 28*). For example, after prolonged periods of activation, microglia become enlarged and no longer able to degrade Aβ they have taken up (*29*). Mutation or loss of TREM2, a cell-surface receptor exclusively of microglia, is strongly linked with impaired functions of microglia, such as breaking down apoptotic cells and digesting Aβ (*30, 31*). The transmembrane receptor CD33, with a 2-fold increased expression in AD patients, not only hinders microglial Aβ uptake and clearance (*32*) but also activates nuclear factor-kappa B (NF-κB) in myeloid cells, upregulating APP productions and contributing to Aβ burden (*33*). Besides, neprilysin, a major enzyme degrading soluble Aβ (monomers and oligomers) in the brain, showed decreased expression and activity in the cortex of elderly AD patients (*34*). Without being effectively removed by brain cells, the mounted Aβ load

could perturb brain microenvironments by impeding ATP formation, leading to mitochondrial dysfunction, ER stress, calcium dysregulation and other neuronal damages (*35*). As innate immunity is highly involved in AD pathogenesis, further studies are needed to examine the specific role of Aβ during an immune activation, such as whether its expression directly leads to inflammation and cognitive impairment.

The Immune Hypothesis of Alzheimer's Disease

In summary, the molecular mechanism of AD is hypothesized to involve the interplay among inflammation, infection and Aβ. Alzheimer's disease is hypothesized to initiate with a pathogen infection, which elicits the activation of microglia and increased production of Aβ as an AMP. The defensive mechanism of the immune system is activated in which microglia release proinflammatory mediators including cytokines and chemokines (*36*) while Aβ oligomers function as AMPs (*37*) or further accumulate in plaques to trap infectious molecules (*15*). If the infection is suppressed, microglia are then in charge of clearing Aβ and cell death, ensuring a healthy brain microenvironment. In contrast, a chaotic situation will progress quickly when infection becomes chronic and reactivates from time to time, resulting in extended releases of pro-inflammatory cytokines or when the mechanism of Aβ clearance is impaired (*29*, *38*). Based on this immune activation hypothesis of AD, our research aims to clarify this relationship among Aβ, inflammation, and AD pathogenesis. Our goals will be to examine whether Aβ aggregation is correlated with inflammation level and if inflammation directly leads to cognitive impairment, one of the most prominent characteristics of AD.

CHAPTER III: *DROSOPHILA* FLIES AS A MODEL ORGANISM

Genetics and Brain Morphology of Fruit Flies Allow for Alzheimer's Research

For decades, the fruit fly *Drosophila melanogaster* have been a useful tool to investigate the molecular mechanisms and pathogenesis of Alzheimer's Disease. The *Drosophila* genome encodes homologs of the human APP, BACE and γ-secretase genes, which similarly produce Aβ-like fragments (*39*). Transgenic expression of human Aβ peptides in flies also resulted in the production of amyloid plaques, neurodegeneration, and behavioral deficits (*40*). This has enabled us to express different human Aβ variants within the nervous system and the fat body, an immune tissue, of *Drosophila* and examine study their effects on molecular and behavioral levels.

Having important brain architectural features in common to humans, fruit flies have been widely used to study learning, memory, and other cognitive functions (*41*). With around 200,000 neurons, *Drosophila melanogaster* is capable of processing visual and olfactory inputs and store them as short-term or long-term memory (*41*, *42*). Specifically, learning or short-term memory in flies, which lasts for second to minutes, is activated in the mushroom body (*41*). From there, information can be stored in the central complex as long-term memory, which resembles mammalian transfer of memory from the hippocampus to the cortex (*41*). Vacuoles in aging fly brains are also indicators of neurodegeneration, which allows for research in brain atrophy, previously mentioned as a prominent feature in AD (*43*). Fruit fly's ability to memorize and display signs of neuron loss similarly to humans allowed us to examine their pathogenesis and cognitive deficiencies. In our study, we imaged the mushroom body and vacuoles in Aβ-expressing flies and developed a cognitive test for fruit flies, which are to be discussed in the next chapters.

Innate Immunity was Examined from Fly-wasp interactions

Drosophila melanogaster has also proven to be an excellent system for identifying highly conserved immune response pathways with both mammals and other insects (*44*, *45*). Fly humoral immune responses to microbial and pathogen infections are characterized by the production of antimicrobial peptides, which are secreted from cells of the fat body into the hemolymph via the Toll, IMD (immune deficiency), and JAK-STAT (Janus kinase/signal transducers and activators of transcription) pathways (*46*). While Toll and IMD mainly respond to bacteria and fungi, JAK-STAT acts as a line of defense against viral pathogens replicating within the host cells (*47*). As Alzheimer's disease has been associated with both bacterial and viral infections mentioned in Chapter II, we were interested in verifying if any inflammation gene in these three pathways showed abnormal gene expression in our Aβ-encoding fly models.

Besides humoral immunity, flies also have the cell-mediated innate immune system, which is activated when hemocytes, or blood cells, react to epithelial damage and foreign molecules. These hemocytes play an important role in sealing epithelial wounds and phagocytizing pathogens and apoptotic cells. In natural *Drosophila* populations, up to 90% of fly larvae have been found to be infected by wasps. To protect themselves, fruit fly larvae trigger the cellular immune response against wasp eggs, called encapsulation. Our preliminary data showed that loss of the APP homolog *APPL* significantly decreases the rate of encapsulation against wasp eggs, suggesting that *APPL* is required to elicit a successful immune response. Further, if this encapsulation response is misdirected against self-tissue, it leads to inflammation phenotypes such as observable melanization of tissues, darker body color, and a more rigid posture with loss of mobility. We also observed that expressing human Aβ42 elicits an inflammation phenotype in fly models following parasitic infection (**Figure 2**). These findings suggested that Aβ might be an immune molecule triggering inflammation upon infection, which can further lead to tissue damage and AD

progression.
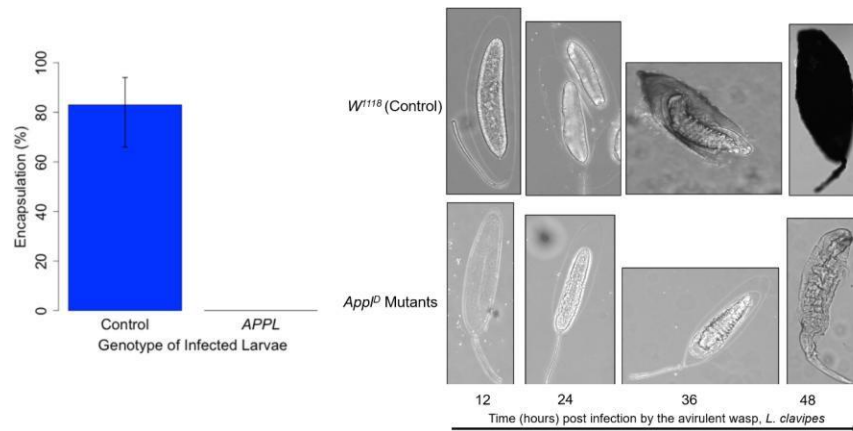
Figure 2: *APPL* mutant larvae had a lower rate of encapsulation compared to control. Unsuccessful encapsulation (lack of melanization) was also observed in *APPL* mutant flies.

Egg-laying Preference Implies Cognitive Functions

Fruit flies are also known to display behavioral immunity to avoid sources of infection (*48*). For example, offspring's fitness and infection risk largely relies on how adult females choose oviposition sites (*49*). In the nature, *D. melanogaster* females prefer decaying fruit to lay their eggs while other species, such as *D. suzukii*, choose to lay their eggs in ripening fruits (*50*). Ethanol proportion in fruits have been measured to be from 0.6% in ripe hanging fruit, to 4.5% in fallen rotten ones (*51*). While flies benefit from consuming fermenting fruits for energy stores and increased longevity, consumption of food with higher than 4% ethanol concentration significantly increases fly mortality (*52*). Interestingly, in the presence of wasps, flies voluntarily choose to lay eggs on 6% ethanol food, which is detrimental to fly fitness, as a form of self-medication against these parasites (*52*, *53*). There is evidence suggesting the involvement of long-term memory in this fly oviposition preference. Neurotransmitters necessary for complex behaviors like learning and memory, such as Neuropeptide F and Dopamine, have a role in *Drosophila*'s preference for egg-laying in alcohol-containing food (*54*, *55*). In addition, long-term memory genes, such as

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dunce (*dnc*) and amnesiac (*amn*) and the transcription factor *Adf1*, required for long-term memory formation, are necessary for flies to show oviposition preference for alcohol food (*53*, *55*). These findings supported that the egg-laying behavior can be used as a tool to examine the cognitive functions of flies. Hence, we developed a Fly Cognitive test to examine fly's ability to sense alcohol, recognize wasps and form memory, which are to be further described in Chapter IV.

CHAPTER IV: RATIONALE AND METHODS

Rationale

The goal of this research is to gain a better understanding about Aβ, inflammation, cognitive defects in the development of Alzheimer's disease. Despite previous studies linking these processes, the mechanisms through which Aβ deposits lead to inflammation and memory impairment are not fully comprehended. The gap in the understanding of this disease led to the focus of this research on aiming to strengthen the relationship among Aβ, inflammation and AD pathogenesis. We hypothesized that inflammation and excessive Aβ load following brain infections contribute to the progression of AD.

The first aim investigated whether Aβ-induced inflammation is associated with cognitive defects, a main characteristic of Alzheimer's disease. With the known associations between Aβ with AD and the immune system, we wanted to test whether Aβ variants have different degrees of inflammation and if that explains differences in pathogenicity in flies. In this aim, we produced fly lines expressing Aβ variants in neurons and examined their cognitive functions, such as their ability to recognize wasps, sense alcohol, and demonstrate a memory-associated strategy to overpower their enemies, as observed in wild-types flies. We also analyzed their brain morphology to look for signs of vacuoles, indicating neuron loss as in Alzheimer's patients. In addition, we also quantified the inflammation levels in these flies using a novel gene expression technology with the help of our collaborators, Dr. Bess Frost and Elizabeth Ochoa at the University of Texas Health Science Center at San Antonio. Our results for this aim suggest that Aβ expression in *Drosophila* resulted in premature neurodegeneration and abnormal inflammation levels.

In the second aim, we wanted to characterize the aggregation characteristics of Aβ variants expressed in flies. We produced fly lines expressing Aβ variants in the fat body, fly's largest immune organ, and quantified protein accumulation using Tris-Glycine gel electrophoresis and

Western blot. We also collaborated with Dr. Michael Webb in the Chemistry department at ISU to test several novel Ruthenium complexes to see if they can eliminate Aβ aggregates in vitro. Our results for this aim suggest that different Aβ variants have different aggregation levels and Ruthenium-based drug could be a potential therapeutic agent for Alzheimer's disease.

Methods

Aim 1: Examining the correlation between Aβ, inflammation and memory defects

1.1. Building fly stocks and crosses

The Q-system is an extensively used method that allows for the expression of transgenes in specific tissues in *D. melanogaster*. This expression system utilizes the binding of a transcription activator (QF) to an enhancer (QUAS) resulting in transcription of downstream responders (*56*). To express a specific gene, flies expressing the transcriptional activator QF in specific tissues were crossed with the strains containing the QUAS enhancer sequence next to the gene of interest. Offspring having both the QF and QUAS transgenes will activate expression of the desired gene.

For our experiments, we used the elav-QF driver to express interested genes pan-neuronal in Aim 1 and the r4-QF driver to express different Aβ variants in the fat body in Aim 2. To produce flies for our experiments, elav-QF or r4-QF flies were crossed with flies of appropriate genotypes (**Table 1**) and kept in 25ºC. The fly stocks yw, elav-QF, amn, dnc and ninaB were obtained from the Bloomington Drosophila Stock Center (NIH P40OD018537), listed in Appendix A.

Table 1: Fly lines used for our experiments

Fly crosses (Driver x Responder)		Descriptions	Control or Experimental
Driver	**Responder**		
elav-QF (Aim 1)	yw	Control fly, does no express any Aβ variant gene	Control
	QUAS- Aβ40	Expressing human Aβ40, the most common and less pathogenic form of Aβ, in the fat body	Control
	QUAS- Aβ42	Expressing human Aβ42, the pathogenic form of Aβ	Experimental
Or	QUAS- Aβ42[F20E]	Expressing human Aβ42 with a "protective" mutation showing decreased aggregation of Aβ peptides	Experimental
r4-QF (Aim 2)	QUAS- Aβ42[E22G]	Expressing human Aβ42 with the "Arctic" mutation showing increased aggregation of Aβ peptides	Experimental
	QUAS- Aβ42[E22G, I31E]	Expressing human Aβ42 with an "arctic" mutation and a "suppressor" mutation. This phenotype still has increased aggregation, but decreased pathogenesis	Experimental
Other fly lines			
amn		Lacking the expression of amnesiac gene that encodes a neuropeptide that functions to prolong medium-term memory. amn mutant flies show defects in memory retention both immediately and 3 hour after training (FlyBase).	Memory mutant control
dnc		Lacking the expression of dunce, which plays a pivotal role in neurological and behavioral plasticity including synaptic development and function, learning and courtship. dnc mutant adult flies show defects in immediate recall memory and short term memory (FlyBase).	Memory mutant control
ninaB		Lacking the expression of neither inactivation nor afterpotential B (*ninaB*) gene, which promotes visual pigment biogenesis in the dark. Unlike control, ninaB mutant flies have visual impairment and do not decrease mating latency in the presence of parasitic wasps.	Vision mutant control

1.2. Examining perceptive functions using fly cognition assay

To examine fly brain functions, including memory, smell, and vision, we optimized the Fly

Cognition Assay based on the protocol of Kacsoh et. al, 2013 (*53)*. Thirty female offsprings from

each genotype were collected about two weeks after eclosion. Next, they were divided into Exposure and Pre-Exposure groups, where flies were exposed to *L. heterotoma wasps* overnight, and Non-Exposure group, where flies were on their own. (**Figure 3**). After the 1-day period was finished, each fly group were transferred into egg-laying corrals to oviposit. Flies of the Exposure group were placed in the corrals along with wasps (**Figure 3-A**) while Pre-Exposure group and Non-Exposure groups did not have wasps in the corrals (**Figure 3-B and 3-C**). These corrals contained two food substrates, including fly instant food mixed with or without 6% ethanol. After one day in the corrals, eggs laid in each food receptacle were counted and recorded. The experiment was repeated ten times for each line of flies. Details of the protocol are listed in Appendix B-1.
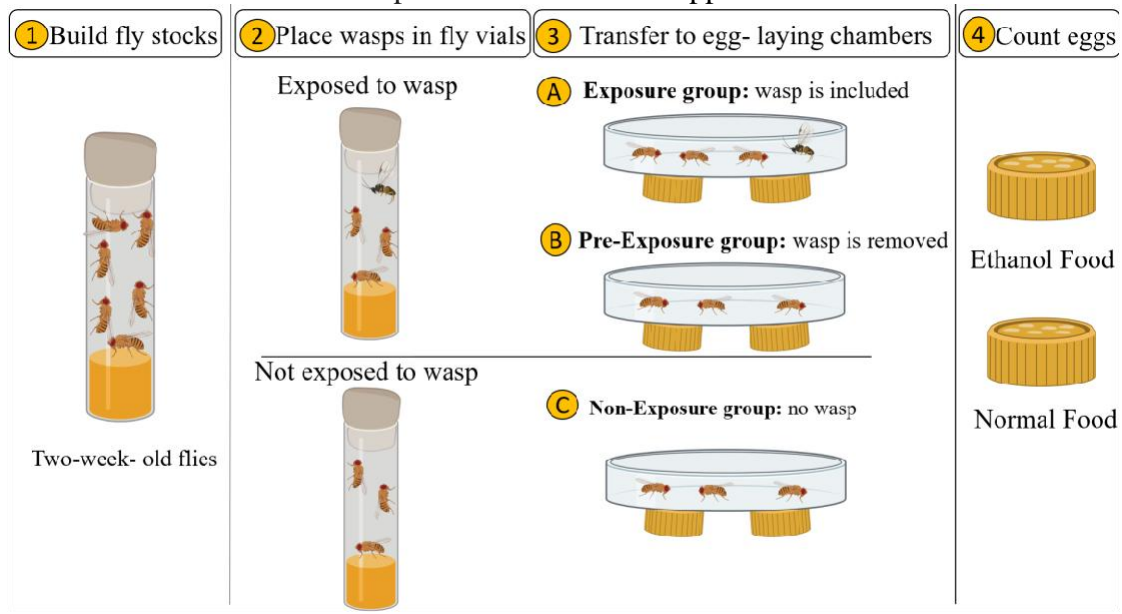
Figure 3: Experimental design for the fly oviposition assay included 4 steps. Following the first step which was building fly stocks and crosses, flies were placed in vials either with wasp or not. After 1 day, they were divided into three groups: Exposure, Pre-Exposure and Non-Exposure and allowed to lay eggs in fly corrals containing 2 food options (6%Ethanol and Non-Ethanol food) overnight. Finally, eggs from each receptacle were counted.

The expected result for the control of this experiment was explained in **Figure 4**. Placed in a fly chamber, flies were allowed to lay eggs on two available substrates, one of which contains fly instant food and water and the other with the addition of 6% ethanol. This 6% amount of alcohol is higher than the preferred amount but not high enough to be lethal to flies. With no external pressure, control flies from Non-Exposure group were expected to oviposit more on the non-ethanol substrate as the other one contains an amount of alcohol unfavorable to flies **(Figure 4-A)**. In contrast, in the presence of wasps, control flies were expected to show an innate behavior to change their oviposition preference toward alcohol food, protecting their larvae from the parasites **(Figure 4-B)**. Flies exposed to wasps a day before also choose to lay eggs on ethanol food in a no-wasp chamber **(Figure 4-C)**. This suggested that they were able to recall previous interactions with wasps.
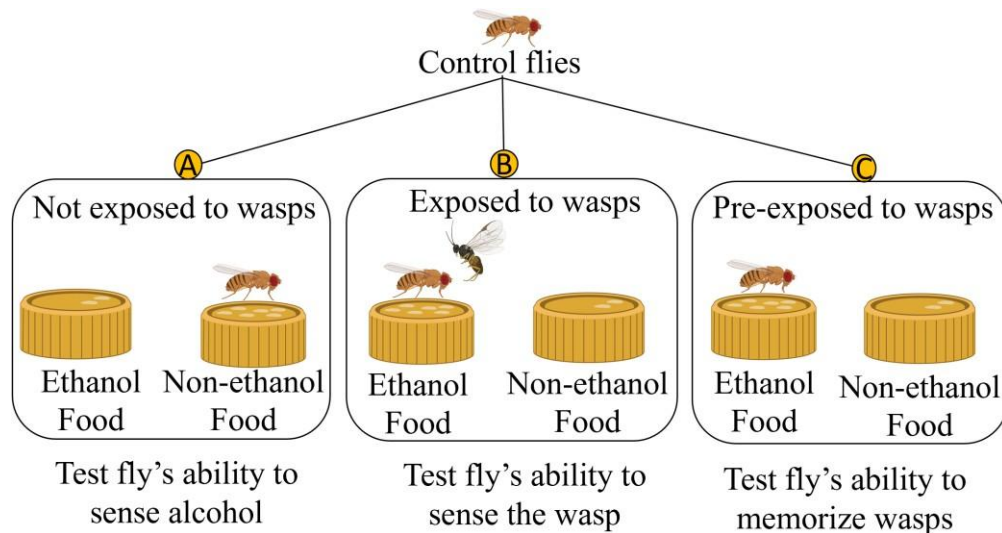
Figure 4: Control flies were expected to oviposit on non-ethanol food in a no-wasp chamber, but incline to ethanol food after exposure with wasps

For this experiment, we examined flies expressing different variants of Aβ in neurons and control flies (yw), which do not express any Aβ variant (**Table 1**). As mentioned in Chapter III, several long-term memory genes, such as dnc and amn, are involved in fly oviposition preference,

making this an ideal test for memory and cognitive functions of flies. Therefore, besides testing Aβ flies, we also included dnc, amn, ninaB mutant flies (**Table 1**) as memory mutant and vision mutant controls in this assay.

1.3. Examining brain morphology via whole mount immunostaining and imaging

The purpose of whole mount imaging was to visualize and quantify neuronal degeneration in fly brains. This method allowed the detection of actin and neuropils by using the phalloidin stain. Most of the brain tissue stains with phalloidin, and except for the stereotypic physiological holes, which usually exist in symmetry (**Figure 5**), the regions devoid of stain were determined as neuron-specific loss of tissues (*57*).

Physiological Holes
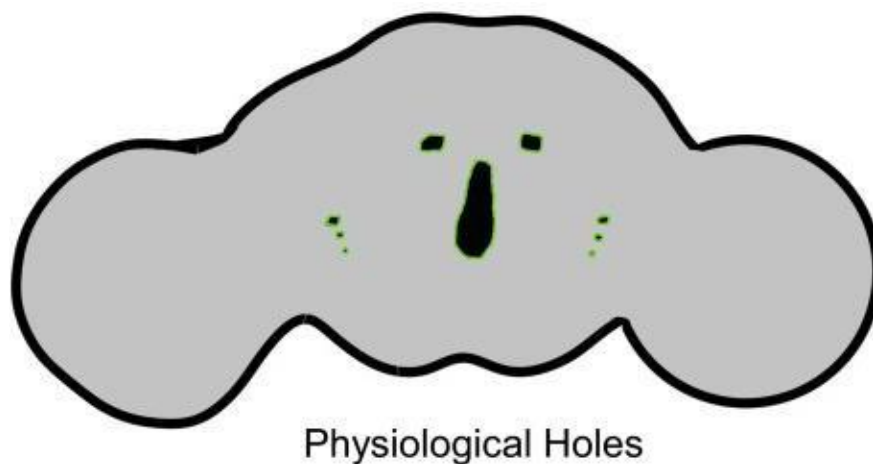
Figure 5: Normally observed vacuoles in fly brains, which are in stereotypical location and almost always symmetrical in nature (*57*)

For the procedure, flies were fixed with 2% paraformaldehyde (PFA) in phosphate-buffered saline containing 0.5% triton X-100 (0.5% PBS-T) overnight at 4°C with nutation. After washing 3 times with 0.5% PBS-T, 5 min each with nutation, fly brains were then dissected in 0.008% PBS-T. The samples were then incubated in phalloidin 633 in 1:1000 dilution for two overnights at 4°C with nutation. After that, the fly brains were washed two times with 0.5% PBS-T, 15 min each and with 1X PBS for 30 min. Next, the brains were pipetted on a microscope slide

which has been attached with a V-cut SecureSeal™ imaging spacer. After all liquid in the sample was aspirated by pipetting, 5μL of Vectashield Antifade Mounting medium was applied on each brain. A cover slip was placed over the spacer and slide containing the brains and sealed with nail polish. The sample was visualized with confocal microscopy and analyzed with Image J software. Using a built-in tracing tool in ImageJ, we went through each sequential frame of the z stack and marked all vacuoles that have a defined enclosure and were oval and spheroid in appearance, which indicated pathogenic holes. A detailed Fly Whole Mount Brain imaging protocol is included in Appendix B-2.

1.4. Preparing RNA extracted samples for Nanostring sequencing

RNA was harvested from 2-week-old adult fly heads using Trizol reagent (Sigma). After being homogenized, the samples were spin at 12000xG for 10 minutes to remove cuticle and debris. Next, as the supernatant was transferred to a new tube, chloroform was added to promote phase separation, allowing RNA to be isolated in the aqueous phase. Isopropanol is then used to precipitate RNA from the samples. The RNA pellet was then washed twice with 70% EtOH and resuspended in 10mM Tris buffer. Additional details of the protocol are included in Appendix B-3.

1.5. Data Analysis and Statistics

Analyses were performed in R. The proportion of ethanol food preference was analyzed using generalized linear models followed by Dunnett's test to compare experimental groups to the control and Tukey's test for pairwise comparisons of all genotypes.

Aim 2: Characterizing aggregation level in different Aβ variants

2.1. Examining aggregation level using gel electrophoresis and western blot

To characterize Aβ aggregation in flies, proteins were first extracted from frozen larvae using RIPA (Radio-Immunoprecipitation Assay) buffer. The protein extraction protocol is further

described in <u>Appendix B-4</u>. Immunoprecipitation was then performed to isolate Aβ peptide from the whole protein extracted samples. Protein G Dynabeads (ThermoFisher) were incubated with 6E10 anti β-amyloid primary antibody (1:2000 dilution) for one hour before added to the protein samples. After a 20-minute incubation, the samples were placed on the magnet, washed 3 times with 1X PBS and eluted with Tris-Glycine loading buffer. Details of the protocol are included in Appendix B-5.

Electrophoresis was completed using 2–20% Mini-PROTEAN® TGX Precast Gels from Bio-Rad, at 100 V for 60 min. The gels were then transferred to a nitrocellulose membrane for 1 hour at 100 V at 4°C, followed by blocking of the membrane in a 3% BSA solution in Tris-buffered saline (TBS) (0.02 M Tris, 0.15 M NaCl, 0.003 M KCl) for 1h. The membrane was incubated in a solution (1:2,000 dilution) of 6E10 anti-Aβ primary antibody (Biolegends) overnight. After washing 5 × 5 min with TBS, the membrane was incubated in a solution containing the secondary antibody (horseradish peroxidase) in 1:10,000 dilution for 3 hours. Last but not least, Thermo Scientific Pierce ECL Western Blotting Substrate kit was used to visualize the Aβ species using a GelDoc imaging system.

2.2. Reducing Aβ aggregation using Ruthenium drug

Metal-based therapeutics for AD have been studied to target aggregates and inhibit fibril formation, since it has been suggested that Aβ peptide showed affinity for metal ions (*58, 59*). Ruthenium-based (Ru) complexes, containing organic ligands bound to the center metal, have achieved great success as anticancer, antiviral and antiparasitic agents (*60, 61*). A number of studies from our collaborator, Dr. Michael Webb, have suggested several Ruthenium(III) derivatives as promising candidates to limit Aβ aggregation and reduce its cytotoxicity (*59, 62–64*). Collaborating with Dr. Webb's lab, we were interested to the effects of four variants of Ru(III) on synthetic Aβ aggregates. Briefly, after a 24-hour incubation with Ru(III) derivatives, Aβ were

evaluated for aggregation using gel electrophoresis and western blot with a similar protocol mentioned above.

Electrophoresis was completed using 2–20% Mini-PROTEAN® TGX Precast Gels from Bio-Rad, at 100 V for 60 min. The gels were then transferred to a nitrocellulose membrane for 1 hour at 100 V at 4°C, followed by blocking of the membrane in a 3% BSA solution in Tris-buffered saline (TBS) (0.02 M Tris, 0.15 M NaCl, 0.003 M KCl) for 1h. The membrane was incubated in a solution (1:2,000 dilution) of 6E10 anti-Aβ primary antibody (Biolegends) overnight. After washing 5×5 min with TBS, the membrane was incubated in a solution containing the secondary antibody (horseradish peroxidase) in 1:10,000 dilution for 3 hours. Last but not least, Thermo Scientific Pierce ECL Western Blotting Substrate kit was used to visualize the Aβ species using a GelDoc imaging system.

CHAPTER V: RESULTS

Aim 1: Examining Cognitive Functions, Brain Morphology, and Inflammation

Cognitive Functions of Control and Negative Control Flies

To test the cognitive functions of flies, we used the Fly Cognitive Assay and divided each fly genotype into three groups. In the Non-Exposure group, flies were not exposed to wasps and allowed to lay eggs in chambers containing two food options: 6% ethanol food and plain food. This group allowed us to test whether flies could sense alcohol and choose to lay the majority of eggs in plain food. Flies in the Exposure group were exposed to wasps for 1 day and allowed to lay eggs in chambers containing wasps. With the wasp included, we wanted to examine whether flies could respond to wasps by changing their oviposition preference and switch to an alcohol food source. Pre-Exposure group are those exposed to wasps for 1 day and laid eggs in corrals without wasps. This is an important group to investigate the memory function of flies, and demonstrated whether flies, which were previously exposed to wasps and not having the wasps around to remind them, could still recall interaction with wasps after one day.

Egg-laying behaviors of control (yw), memory mutant (amn and dnc) and vision mutant (ninaB) flies were demonstrated in **Figure 6**. Control flies (yw) successfully displayed an egg-laying preference as we had expected in **Figure 4**. There was a majority of eggs laid in plain food when wasps were not present in the Non-Exposure group. However, after fly's exposure to wasps, most eggs were found in ethanol-laden food, both when wasps were around and excluded from the corrals. Flies losing important memory formation genes, such as *dnc* and *amn*, were able to sense alcohol by ovipositing on non-ethanol food when unexposed to wasps (Non-Exposure group), successfully switched to alcohol in the presence of wasps (Exposure group), but not able to maintain ethanol preference in the Pre-Exposure condition. This suggested that our Fly Cognitive

Test was successful in detecting the cognitive impairment of these two memory mutant controls. Interestingly, vision mutant flies (ninaB) were able to choose ethanol after exposing to wasps (Pre-exposure group), yet showed no interest in ethanol food when wasps are around (Exposure group).
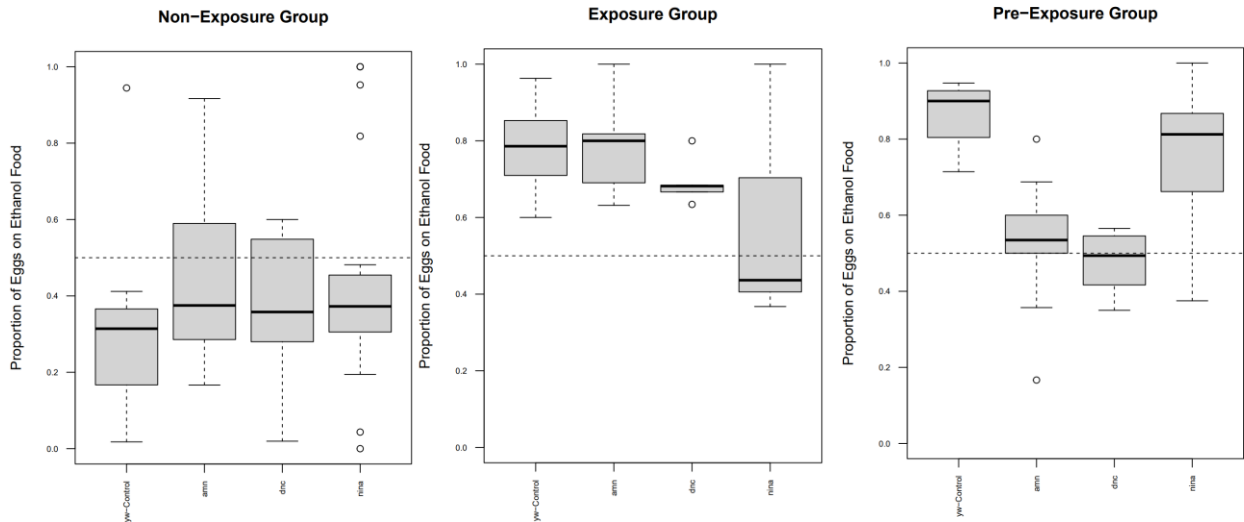
Figure 6: Oviposition preference of control (yw), memory mutant flies (amn and dnc) and vision mutant flies (ninaB). All flies in Non-Exposure group (left panel) was able to avoid ethanol-containing food and lay the majority of eggs on the plain food receptacle. Control (yw) and two memory mutant flies in the Exposure group (middle panel) switched to ethanol-laden food while ninaB failed to do so. In the Pre-Exposure group (right panel), control and ninaB showed preference in ethanol food, while two memory mutant flies did not.

Aβ42 and Their Variants Have Different Detrimental Effects on Fly Cognitive Functions

Next, flies expressing different Aβ variants were examined in this assay, demonstrated in **Figure 7**. We were interested in investigating flies expressing non-pathogenic (Aβ40) and pathogenic (Aβ42) Aβ variants. We also tested three other Aβ42 variants with the "Arctic" mutation leading to an increased Aβ aggregation (Aβ42[E22G]), the "protective" mutation having less aggregation (Aβ42[F20E]), and the "Arctic" and "suppressor" double mutations (Aβ42[E22G, I31E]). In the Non-exposure group, the control and all Aβ flies were able to show preference for

non-ethanol food over ethanol food, suggesting that they were able to sense and dislike ethanol food (**Figure 7**, left panel). On the other hand, in the presence of wasps, all flies prefer laying eggs in ethanol food except for Aβ42[E22G] flies, suggesting this Aβ variant causes an effect on the flies' ability to perceive wasps (**Figure 7**, middle panel). In the Pre-exposure group, while the control (yw) and Aβ40 flies successfully recalled wasp exposure, all four Aβ42 variants did not show preference toward alcohol, indicating their impaired memory functions (**Figure 7**, right panel).
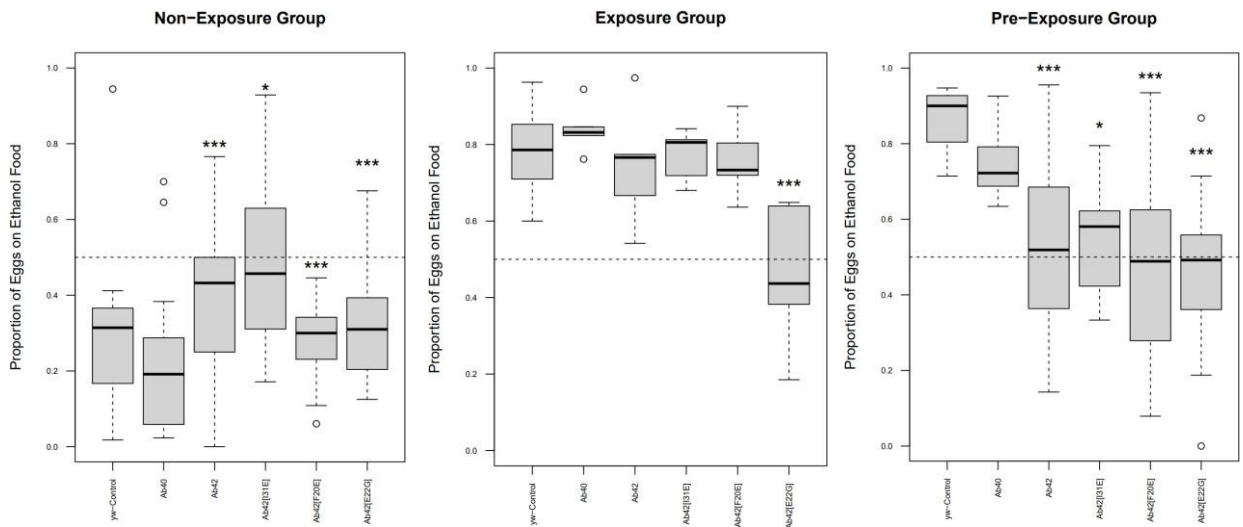
Figure 7: Flies expressing different Aβ variants demonstrated different pathological effects. In the Non-Exposure group (left panel), all flies showed oviposition preference toward the non-ethanol food. On the other hand, all genotypes in the Exposure group (middle panel), where flies had been exposed to wasps a night before and allowed to lay eggs in wasps-containing corrals, switched into ethanol-laden food, except for flies with the Arctic mutation, Aβ42[E22G]. From the Pre-Exposure group (right panel), which was exposed to wasps but did not have wasps in the egg-laying corrals, only control (yw) and Aβ40 were able to maintain a strong preference to ethanol food and all four Aβ42 variants in this group were not. ***P < 0.001, **P < 0.01 and *P < 0.05 illustrate the remarkable difference of each genotype in comparison with the control.

Aβ42-Expressing Brain Showed Signs of Neurodegeneration

As cognitive defects were observed in the Aβ42- expressing flies, we wanted to verify if there was any difference in these brains' morphology. Brain atrophy, or the loss of neurons and connections between neurons, has been associated with aging, dementia and infectious diseases (*65*). Whole mount brain imaging was performed with the aim to detect cavities or spaces in the brain associated in neuron loss. Our results showed that Aβ42 and Aβ42[E22G] brains had a greater number of vacuoles and larger average vacuole size compared to the control (yw) and Aβ40 brain samples (**Figure 8, 9, 10, 11**).
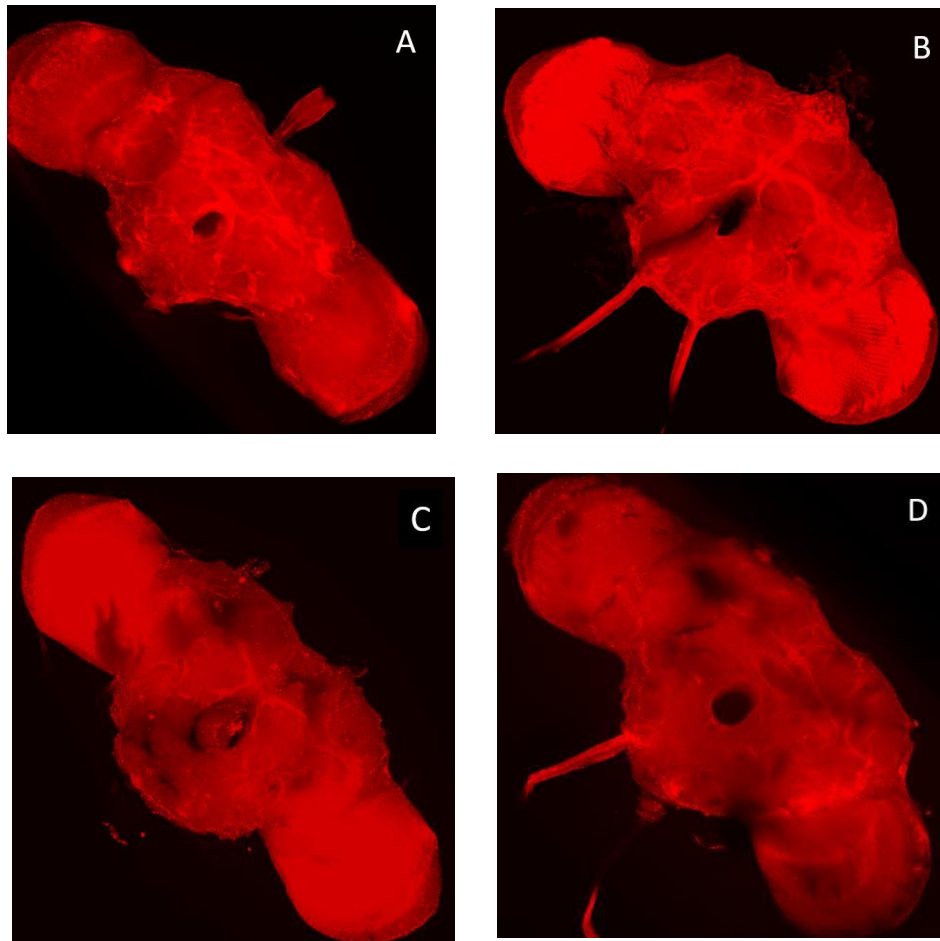
Figure 8: z-stack images of control (yw) (**A**), Aβ40 (**B**), Aβ42 (**C**) and Aβ42[E22G] (**D**) brains, 4 weeks old. (**A**) and (**B**) yw and Aβ40 brains showed no significant dark spots and vacuoles (dark areas devoid of stains in the brain. (**C**) and (**D**) The degree of vacuolization (enlargement

and number of vacuoles) in Aβ42 and Aβ42[E22G] brains increased, in which many vacuoles were observed.
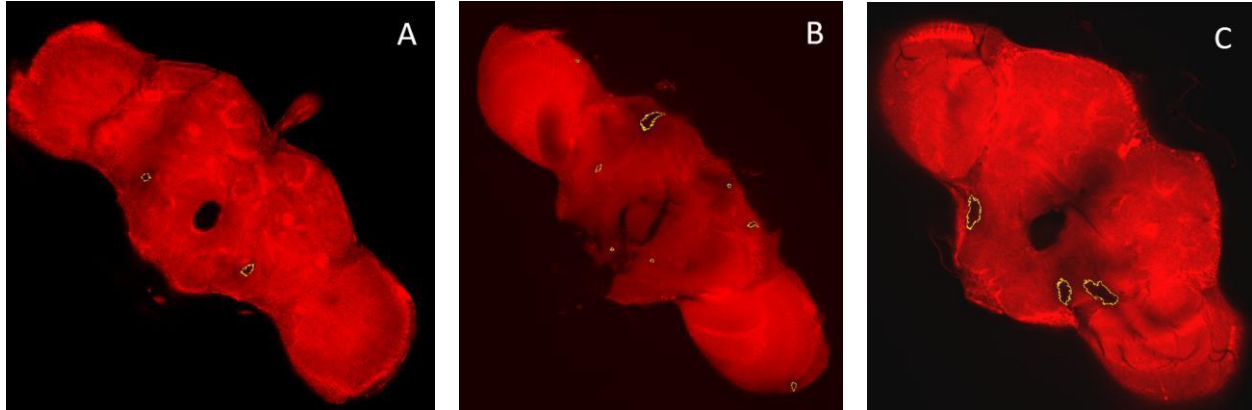
Figure 9: Cross sections of control (yw) (**A**), Aβ40 (**B**) and Aβ42 (**C**) brains, 4 weeks old. Vacuoles were marked with yellow outlines using the tracing tool in ImageJ. (**A**)While a few vacuoles were observed in the control (yw) brain, they were present in symmetry, indicating stereotypical physiological holes. (**B**) Aβ40-expressing brain demonstrated several small vacuoles. (**C**) Many vacuoles at different locations were observed in Aβ42 brain with significantly increased area sizes.
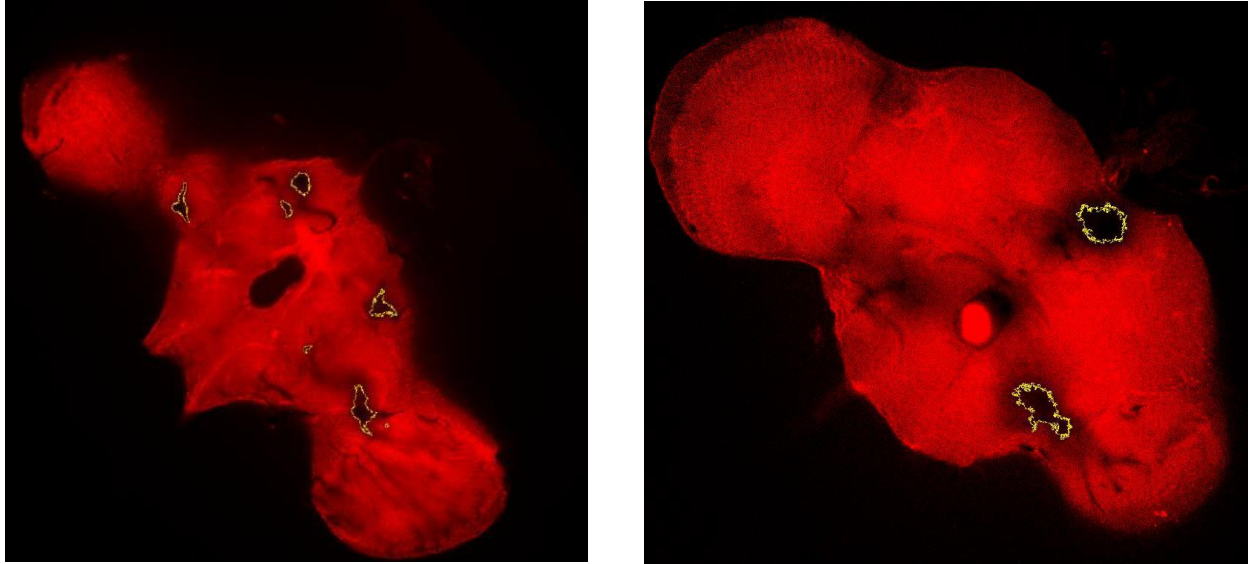
Figure 10: Two cross sections of two different Aβ42[E22G] fly brains, 4 weeks old. Yellow outlines represented vacuoles. Brains from this genotype showed a significantly increased number of dark areas and enlargement of vacuoles.
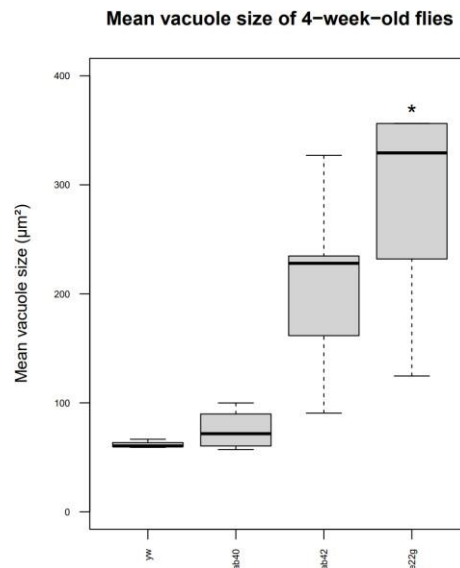
Figure 11: Mean vacuole size (in µm²) in the brain of 4-week-old flies including control (yw), Aβ40, Aβ42 and Aβ42[E22G]. The data was based on the imaging of three yw, four Aβ40, five Aβ42 and five Aβ42[E22G] brains. *P < 0.05 illustrates the remarkable difference in comparison with the control.

Abnormal Inflammation Levels of Aβ Flies Using Nanostring Sequencing

As signs of neurodegeneration and cognitive impairment were observed, we wanted to verify if inflammation plays any role in these defects. Specifically, we examined a number of inflammation genes involved in toll, IMD and JAK/STAT pathways (**Figure 12**). Interestingly, there were genes showing an increased expression when comparing non-pathogenic Ab flies (Aβ40) to the pathogenic Aβs (Aβ42 and Aβ42[E22G]). For example, in Toll pathway, *IM* genes (molecules induced by infection) showed stronger expression in pathogenic Aβ flies. In IMD pathway while *imd* and *fadd* (fas-associated death domain) have increased transcript abundance in pathogenic flies. Of the Jak/STAT pathway, many genes that have a stronger expression in pathogenic flies are involved in cytokine receptor activity and protein binding (such as *upd1, upd2, upd3* and *dome*). The full list of inflammation genes examined is included in Appendix C.
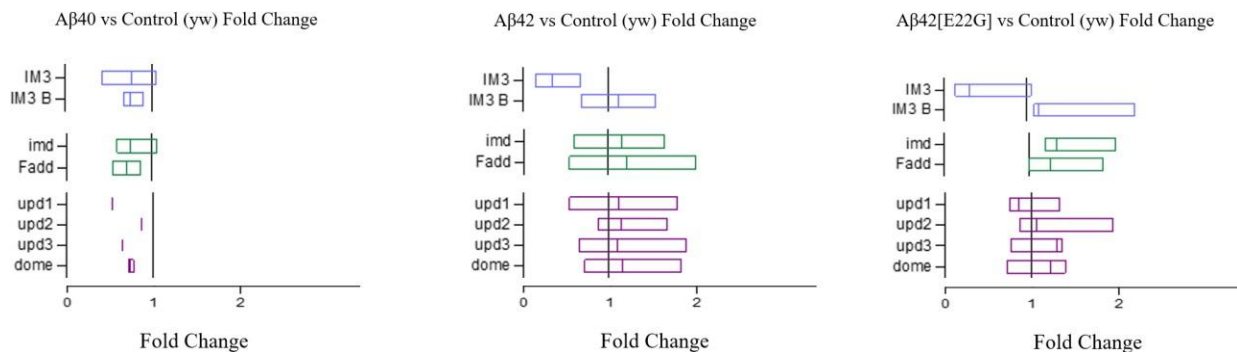
Figure 12: Nanostring sequencing showed different expression of inflammation genes. Blue = Toll, green = IMD and purple = Jak/STAT. RNA samples were extracted from 2-week old female fly heads. Each graph showed the gene expression fold change compared between control and each genotype of interest (Aβ40-left panel, Aβ42-middle panel and Aβ42[E22G]-right panel). There was an increased trend in the transcript abundance of several inflammation genes in the pathogenic phenotypes (Aβ42 and Aβ42[E22G]) compared to non-pathogenic one (Aβ40).

Aim 2: Characterizing Aβ Aggregation and Testing a Therapeutic Agent

Aβ Variants Accumulate as Oligomers in Flies

Aβ42 and the Arctic mutation Aβ42[E22G] accumulated as oligomers and accumulated differently across tissues. The less pathogenic variant Ab40 appeared to be cleared more efficiently. Aβ42 was expressed with highest level in the fat body, an immune tissue of flies, while the E22G oligomers show decreased accumulation.
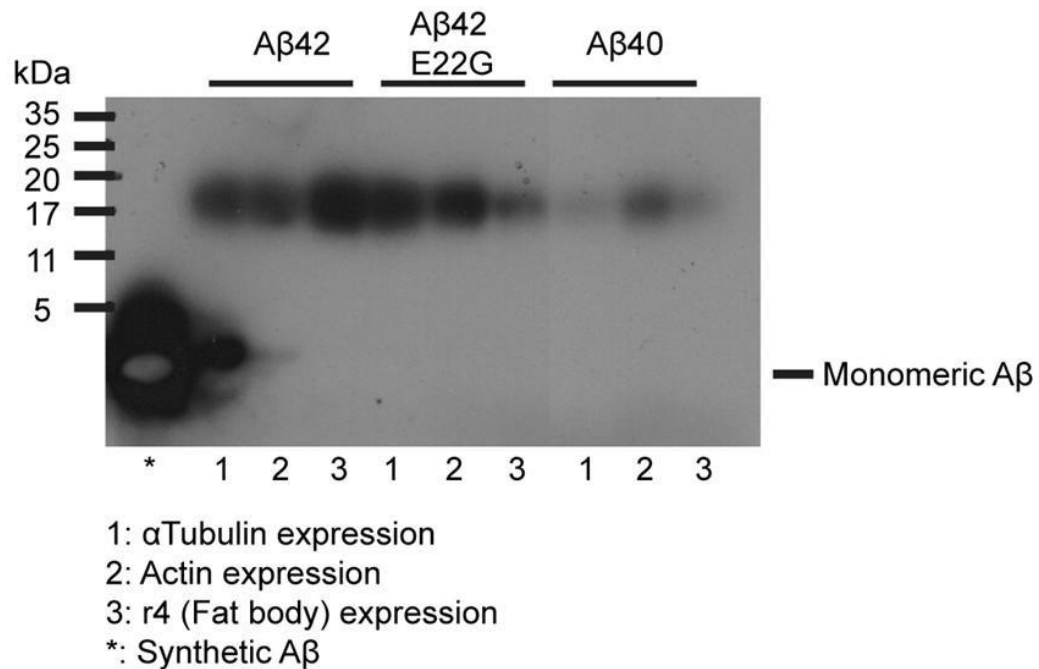
Figure 13: The result of Western Blot analysis showed Aβ accumulation in different fly tissues. αTubulin- marked as 1, actin- marked as 2, and fat body- marked as 3. While synthetic Aβ (lane *) accumulated around 3-4kDa, all Aβ-expressing larval samples showed protein bands around 17-20kDa, suggesting Aβ aggregating into oligomers.

Reducing Aβ Aggregation using Ruthenium Drug

Gel electrophoresis and Western Blot results suggested that among the four Ruthenium-based complexes we tested, Ruth4 had a positive effect on reducing aggregates. Aβ aggregates were showed to accumulate in vitro around 150kDa.

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Figure 14: Ruthenium complexes show different effects on reducing synthetic Aβ aggregation. Four different Ruthenium-based complexes were examined for their ability to break down synthetic Aβ aggregates. Western Blot analysis suggested that Ruth4 drug was the most effective in reducing Aβ aggregates, which accumulated around 150kDa in vitro.

CHAPTER VI: DISCUSSION

Aβ42 Expression is Associated with Cognitive Impairment

Our findings suggested the association among inflammation, Aβ expression and cognitive defects. First, by using Fly Cognitive Assay, we demonstrated that the expression of pathogenic Aβ variants in *Drosophila* brains was linked with cognitive impairment. Among the five Aβ variants, Aβ40, the non-pathogenic peptide, was the only variant that passed the three cognitive criteria in this assay: ability to sense wasp, recognize alcohol and recall previous memory formed after 1-day exposure with wasps. Flies expressing Aβ42 and Aβ42[E22G] showed impaired cognitive functions such as memory impairment and unable to recognize wasps respectively (**Figure 7**). The Aβ42[E22G, I31E] flies, which had the I31E suppressor mutation to decrease pathogenesis, showed a slightly better performance than the other Aβ42 variants (**Figure 7**, Pre-Exposure group). Statistical analysis also suggested the remarkable difference when pairwise comparing between each pathogenic variant and the control (**Figure 7**).

The pathogenic of flies expressing Aβ variants was also supported when we measured the degree of vacuolization in fly brains by counting number of holes and measuring their average sizes. Using a tracing tool in ImageJ software, within each sequential frame of the z stack, we marked any region devoid of stains, oval or spherical in appearance and had a defined enclosure with yellow outlines. Control (*yw*) and Aβ40 flies were observed to have several holes with negligible sizes, some of which are present in symmetry in the brain, indicating stereotypic physiological vacuoles due to air sac in the brain (**Figure 8**). On the other hand, Aβ42 flies, especially the ones expressing the Arctic mutation variant, Aβ42[E22G], demonstrated pronounced enlargement of vacuoles, indicating neuron loss in the brain. (**Figure 9, 10 and 11**). These results further supported our findings in Aim 1.1 that flies expressing pathogenic variant Aβ42 and Aβ42[E22G] had impaired cognitive functions and altered brain morphology.

In **Figure 11**, ANOVA analysis additionally suggested that the mean sizes of vacuoles varied by genotype (p = 0.0311). Analysis via Dunnett's Test also suggested a remarkable difference between Aβ42[E22G] and control (yw) (p = 0.0324). Tukey's Test was also performed to make pairwise comparisons among genotypes, however, only the pair Aβ42[E22G] and Aβ40 had a significant difference (p=0.0467). This could be due to the insufficiency of our data as only three yw, four Aβ40, five Aβ42 and five Aβ42[E22G] brains were analyzed in this graph. More replicates are needed to substantiate the result of this experiment.

A few inflammation genes are upregulated in cognitive impaired flies

As we quantified the transcripts abundance of selected inflammation genes involved in the three main immune pathways in flies, we observed that a few important genes were present in an increased amount in pathogenic flies (**Figure 12**). Several immune- induced peptides playing a role in resisting bacterial and fungal infections (*IM3* and *IM3B*) showed a significantly higher level in Aβ42[E22G] flies. In addition, inflammation genes involved in defense response to bacterium and death receptor binding activity (*imd* and *Fadd*), also showed increased transcript abundance in pathogenic flies. We also observed increased expression in genes associated with cytokine receptor activity and protein binding (*upd1, upd2, upd3 and dome*). These results suggested that the expression of pathogenic Aβ variants (Aβ42 and Aβ42[E22G]) was linked to increased expression in several inflammation genes while Aβ40 expression did not induce any significant effects on the immune pathways. This experiment allowed us to identify possible key genes associated with AD pathology and neuroinflammation, which improved our understanding about mechanisms leading to AD.

Aβ Variants Accumulate Differently in Flies

Besides, we observed that there were some differences in the aggregation levels among Aβ variants (**Figure 13**). Aβ40 was observed to oligomerize the least across tissues when compared

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to the other pathogenic variants, Aβ42 and Aβ42[E22G]. In the fat body, an immune tissue of flies, Aβ42 was expressed with even higher level than E22G. This suggested that the aggregation characteristics of the Aβ variants might have a role in determining whether it is involved in the immune system. Follow-up studies are needed to further characterize Aβ aggregates formed by different Aβ variants, and investigate if any factor, such as their structures, shapes, and binding ability, affects their function in the immune system.

Ruthenium-based Drug Could Be a Potential Treatment for AD

Aβ aggregates, known as amyloid plaques, have been observed in all AD patients and are linked with pathogenesis of this disease. The understanding of different Aβ variants in this study has allowed us to further investigate the effect of novel Ruthenium-based complexes on reducing Aβ aggregation as a potential therapeutic treatment. Result from western blot analysis showed that Ruth4 was the most efficient in breaking down Aβ aggregates and might be a potential agent for future studies, such as testing the effects of this drug in flies.

CHAPTER VII: FUTURE DIRECTIONS

Testing other Sensory Functions of Flies

When we tested vision mutant flies, ninaB, we observed that they were inclined to lay eggs on ethanol surface after 1 day interacting with wasps similarly to controls, suggesting vision defects was not associated with memory process in the Fly Cognitive Assay. As vision and olfactory inputs are two primary sensory sources in flies (*42*), we hypothesized that olfactory defects in Aβ- expressing flies might be the reason why they could not form memory and show expected oviposition preference. We are interested in using Y-maze assay to test long-term associative olfactory memory of flies expressing Aβ variants.

Examining How Variants Form Aggregates

The western blot result suggested that Aβ42 was expressed in an immune tissue with a higher level than Aβ42[E22G] (**Figure 13**). Furthermore, in our Fly Cognition Test, flies carrying a protective mutant, Aβ42[F20E], were not able to show intact memory function in the Pre-Exposure group, despite performing similarly to controls in Non-exposure and Exposure groups (**Figure 7**). To further understand why the protective Aβ42[F20E] variant still caused damage to the brain, we plan to monitor how all variants form aggregates by using Thioflavin T fluorescence spectroscopy and transmission electron micrography, as suggested in this study by Brorsson et. al, 2010 (*66*). By comparing the nature of aggregate formation between Aβ variants, it will help to understand if the shape and structure of the aggregate determined its neurotoxicity.

Testing the Efficiency of Ruthenium on Reducing Aβ Accumulation in Flies

As Ruthenium was shown to reduce synthetic Aβ aggregation, we wanted to examine its effects on the Aβ-expressing flies. Briefly, we plan to perform a protein extraction on fly samples, from which Aβ will be immunoprecipitated following the protocol in Appendix B-5. Then, these Aβ samples will be incubated overnight to stimulate the formation of aggregates before being

treated with a Ruthenium-based complex. After a 24-hour incubation, samples will be separated on gel electrophoresis followed by a western blot. If this Ruthenium agent can modulate Aβ aggregation, we expect to see bands with lower molecular weight and reduced intensity when comparing the treated with the untreated samples. In addition, we are also interested in testing an appropriate dilution of this drug on live flies and use our Fly Cognition Test to examine whether flies expressing Aβ variants have improved cognitive functions compared to no-treatment controls. This will contribute to the advancement of novel therapeutic treatments for Alzheimer's disease.

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APPENDIX A: TABLE OF GENOTYPES

Short name	Stock Number (Bloomington *Drosophila* Stock Center)	Gene components
yw	1495	y[1]w[1]
ninaB	24776	w[*]/Dp(1;Y)y[+]; ninaB[1], P{w[+mC]=UAS-ninaB.G}3
amn	5954	amn[1]
dnc	6020	dnc[1]
elav	66466	P{w[+mW.hs] RFP[mCh.3xP3.cPa]=ET-QF2.GB}elav[C155-QF2]; betaTub60D[Pin-1]/CyO

APPENDIX B: DETAILED PROTOCOLS

1. Fly Cognition Assay

Day 0: Grow fly/wasp stocks

- Keep Wild-type, and experimental flies on standard *Drosophila* medium and *Leptopilina heterotoma* wasp on standard wasp medium

- Collect virgin female flies and male flies and make crosses if needed

- Flies were aged together and were 14 days old (post-eclosion) by Day 1 of the experiment

Day 1: Exposure to wasps

- Once flies are ready, collect 30 females from each genotype and divide them into 3 groups, exposure, pre-exposure and non-exposure:

    o For exposure and pre-exposure groups: add 10 flies and 3 female wasps in each 1X food vial

    o For non-exposure group: put 10 flies in each 1X food vial.

- These vials will be placed at room temp, overnight

Day 2:

Prepare food substrates

- Prepare the blue Drosophila media in two batches, 0% and 6% ethanol food

- Measure about 0.375 g* of Instant Blue Drosophila medium and place into the receptacles (caps of 15 mL Falcon Tubes) (* the amount can be approximate)

- For 0% ethanol food: add 2250 µL of distilled water onto instant blue Drosophila media and allow it to be absorbed.

- For 6% ethanol food: add 1650 µL of distilled water to instant food, add 140 µL of 95% ethanol, then add 460uL water on top

Place flies into fly corrals:

- For exposure: Divide 20 flies into 2 group: Exposure (with wasps) and Pre-exposure (without wasps) and placed them in corrals containing the 2 food substrates

  o For the exposure group: 10 females + 3 females wasps.

  o For the pre-exposure group: 10 females

- For non- exposure: Place flies (10 females) in corrals containing the 2 food substrates

- Affix receptacles to the fly corrals using standard laboratory tape

- Place the corrals in a 25° C environmental chamber (12:12 light/dark cycle and ~30% relative humidity) and allow flies to oviposit

- After 24 hours, remove the food receptacles from the fly corrals

- Count the number of eggs that had been laid in each food type under the microscope and record

2. Fly Whole Mount Brain Imaging

Day0: Prepare fly stocks+ Reagents

Prepare:

- 2% PFA in 0.05%Triton-X: dilute 4%PFA in 1:1 ratio with 0.5% PBS-T

- 0.5% PBS-T

- 995mL 1X PBS +5mL Triton X-100

- 0.008% PBS-T

- Stains: Dilute in recommended concentration

  o 633 phalloidin: 1:1000

Day 1: Fix flies

Prepare:

- o   2 mL Eppendorf tubes

- o   2% PFA in 0.05%Triton-X

- o   P-1000 and tips

1. Put flies on CO2

2. Transfer flies to pre-labelled 1.5 mL Eppendorf tube (fewer than 15–20 flies/ tube)

3. Add 1 mL of 2% PFA in 0.05%Triton-X and invert closed tubes

4. Incubate tubes on a nutator, rocker or shaker at 4C overnight

Day 2: Dissect brains and stain

Prepare:

- o   0.008% PBS-T, 0.5% PBS-T, 1X PBS

- o   Stains: Phalloidin /DAPI

- o   Eppendorf tubes

- o   P-1000 and tips

- o   Dissecting tools

5. Wash flies: Slightly angle tube, aspirate the top layer of liquid, then replace with 1 mL 0.5%

PBS-T, wait 15 min each with nutation -> repeat 4 times

Note: Prior to brain dissections, fixed whole flies can be stored in $1\times$ PBS at 4°C for up to

several days. Dissected brains can also be stored in $1\times$ PBS at 4°C for several days.

6. Prep new tubes containing 600uL 0.008% PBS-T and dissect fly brains in 0.008% PBS-T

7. Stain in phalloidin 633 in appropriate dilution for 2 overnights at 4°C in nutation

Day 4: Mount flies on microscope slides and imaging

Prepare:

- o Microscope slides, coverslip, nail polish

- o Antifade mountant

- o Brush, P-1000 and tips (prepare some cut tips to transfer brains)

10. Wash 2x15min in 0.5% PBS-T and then wash in 1X PBS 30min

11. Mount fly brains anterior side up on microscope slides

- Using a brush, position fly brains at a 45 angle with anterior side facing up

- Add 30uL of mountant on the top portion of the imaging spacer

- Slightly angle the cover slip, and gently place over wells containing the fly brains.

- Seal perimeter of coverslip with nail polish and allow nail polish to dry before storing or imaging

12. Use confocal microscopy to image

- Set resolution of 2048 x 2048 with a Z-step of 1mm

13. Image J analysis:

- Using the wand (tracing) tool within Fiji, isolate vacuoles at their largest area and assign them as regions of interest (ROIs).

- Adjust tolerance as needed within the tool to best select each vacuole.

- Within the ROI manager, save each set of ROIs per brain as its own ''.zip'' file.

- Upon completion of the stack, select all ROIs per given brain and perform Measure (with Area selected within Set Measurements) and save Results as ''.csv''. Import measurement data within preferred stats package

3. RNA Extraction with TRIzol

Sample Prep:

Freeze ~10 flies/ microtube in -80°C in advance

Day 1

- Prepare two new microtubes/ sample

- TRIzol (TRI reagents), Chloroform and Isopropanol

- Toxic Waste Container

- Pipettes and tips

1. Add TRIzol to sample microtubes to lyse cells (approx 2X sample volume: **eg: 200uL**)

2. Homogenize the sample with the hand homogenizer

3. Spin at 12000 xG for 10 minutes at 4°C (Remember to orient the microtube properly and add balancer tube if needed)

4. Transfer cleared homogenate (top layer) to a fresh tube (pellet of debris, lipid layer (white layer on top) left behind)

5. Incubate for 5 minutes at RT

6. Add 0.2 ml chloroform/1 ml of trizol **(eg: 40 uL)**

7. Shake the microtubes by hands for 15 seconds and incubate 3 minutes at RT

8. Spin at 12000 xG for 15 minutes at 4°C

9. Transfer RNA (aqueous phase, top layer) to a fresh tube

10. Precipitate the RNA by mixing 0.5 ml isopropanol/1 ml trizol **(eg: 100uL)**

11. Store overnight/ several days in -20C

Day 2

Prepare

o Prepare 70% EtOH, 10mM Tris pH7.5

o Toxic Waste Container

o Pipettes and tips

12. Spin at 12000 xG for 15 minutes at 4°C

13. Remove the supernatant (top layer)

14. Wash the pellet once with 70% EtOH (1 ml/1 ml of trizol) **(eg: 200uL)**

15. Spin at 12000 xG for 15 minutes at 4°C

16. Remove the supernatant

17. Briefly air-dry (5-10 minutes)

18. Resuspend in ~25uL 10mM Tris pH7.5

19. Incubate several hours/ overnight in 4C and store in -20C

4. Protein Extraction

<u>Prepare:</u>

- Ice bucket

- RIPA buffer

- Homogenizer and pestle

- Prepare 1 new tube per sample

<u>Protocol</u>

1. Freeze all samples @ -80C.

2. Add 150 µL ice-cold RIPA lysis to larvae

3. Homogenize with an electric homogenizer on ice.

4. Wash the pestle with an additional 150ul µL ice-cold RIPA lysis buffer.

5. Agitate the contents for 2 h at 4°C. (on the nutator)

6. Centrifuge at 13,000 x g for 20 minutes at 4°C.

7. Carefully collect the supernatant containing the soluble protein and place in a new tube, kept on the ice. Discard the pellet.

8. Freeze at -20C

5. Immunoprecipitation of Aβ

<u>Prepare:</u>

- Dynabeads

- Magnet

- Antibody (6E10)

- 1X PBS (50mL 10X PBS + 450ml H20)

- 1X PBS with 0.02%Tween 20 (10mL 1X PBS + 200uL)

- Elution buffer

- 2 new tubes/ each sample

<u>Separate Dynabeads from buffer:</u>

- Completely resuspend Dynabeads® by pipetting (2 minutes).

- Transfer 50 µl (1.5 mg) Dynabeads® to a tube.

- Place the tube on the magnet to separate the beads from the solution and remove the supernatant.

- Remove the tube from the magnet.

<u>Binding of Antibody </u>(allow antibodies to bind to beads)

- Make antibody dilution by adding 1 ul 6E10 anti β-amyloid primary antibody (1mg/ml) to 10ml PBS with 0.02% Tween20

- Add 10000uL antibody to the tube containing beads and shake well

- Incubate with rotation for 1 hour at room temperature.

- Place the tube on the magnet and remove the supernatant.

- Remove the tube from the magnet and resuspend the beads-Ab complex in 200 µl PBS with 0.02%Tween 20. Wash by gentle pipetting.

<u>Immunoprecipitation of Target Antigen </u>(beads capture protein of interest)

- Place the tube on the magnet, aspirate supernatant, remove from magnet.

- Add 500uL of the sample containing the antigen (Ag) and gently pipette to resuspend the Dynabeads®-Ab complex. (we used 100uL)

- Incubate with rotation for 1 hour at room temperature to allow Ag to bind to the Dynabeads®-Ab complex.

- Place the tube on the magnet. Aspirate supernatant, remove from magnet

- Add 200 µl 1X PBS to wash the Dynabeads®-Ab-Ag complex. Gentle pipetting to resuspend. Aspirate supernatant, remove from magnet

- Repeat step 5 twice more (3 wash total)

- Resuspend the Dynabeads®-Ab-Ag complex in 100 µl PBS and transfer the bead suspension to a clean tube. This is recommended to avoid co-elution of proteins bound to the tube wall.

Elution of Target Antigen (remove proteins of interest from beads)

- Place tubes on the magnet. Aspirate off supernatant.

- To elute the protein off the beads, add 20ul elution buffer

- Incubate with rotation for 20-40 minutes at 4C

- Place tubes on the magnet (ensure all beads are on the magnet)

- Transfer supernatant to new tube

- Nanodrop

- Store @ -20C (we stored @ -80C)

APPENDIX C: TRANSCRIPT ABUNDANCE OF INFLAMMATION GENES

Below is the graph demonstrating the full list of inflammation genes via Nanostring gene expression.

Fold change compared to YW (control) at 1