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A novel approach to study the effects of the high-fat diet on markers of Alzheimer's disease in a genetically stratified Drosophila population

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Contents

Abstract

A high-fat diet is emerging as a particularly influential risk factor for Alzheimer's disease due to the strong association with gut dysbiosis, inflammation, oxidative and metabolic stress, and cognitive deficits. We suggest that disturbed cholesterol biosynthesis and metabolism are one of the major factors through which the diet can exert its influence on the markers of neurodegeneration. The blood-brain barrier (BBB) is an important protective mechanism which helps maintain cholesterol homeostasis and diet-induced oxidative stress is associated with its damage. We investigated the effects of the high-fat diet on these processes by assessing the brain cholesterol pool, lipid peroxidation levels, and morphological neurodegeneration of Drosophila mutants with familial and sporadic genetic predisposition. We also adapted two additional protocols which will be useful in further investigation of the role of high-fat in Alzheimer's pathology: 1) assessment of the BBB integrity and 2) testing of the short- and long-term memory deficits using a T-Maze manufactured using a 3D Printer. Our findings demonstrate that the high-fat diet disturbs cholesterol homeostasis in both mutant groups, but lipid peroxidation and neurodegeneration are characteristic of familial mutants that present with much earlier disease onset.

Background

1. Alzheimer's Disease

1.1 Epidemiology

Alzheimer's disease (AD) is a neurodegenerative disorder that has been declared a global health challenge with a growing number of people affected, economic burden, and social impact, as stated by the World Health Organisation (WHO, 2021). Since its initial description by Dr Alois Alzheimer in 1907, this neurodegenerative pathology has been widely recognised for its detrimental symptoms and impact on the quality of life. An estimated 43.8 million dementia cases have been recognised in 2016 compared with 20.3 million cases 16 years earlier (GDB 2016 Dementia Collaborators, 2019). Furthermore, it is predicted that the number will increase to 152 million people with dementia diagnosis by 2050 (GDB 2016 Dementia Collaborators, 2019)).

Alzheimer's disease is believed to be the most common form of dementia as it accounts for 60 to 80% of all dementia cases (Barker et al., 2002). Other prevalent causes of dementia include Lewy body dementia, Parkinson's disease, and vascular dementia. Age is the major risk factor for the disease onset. The prevalence and the overall burden were found to positively correlate with the growing life expectancy and ageing population (Li et al., 2022) (Fig. 1).

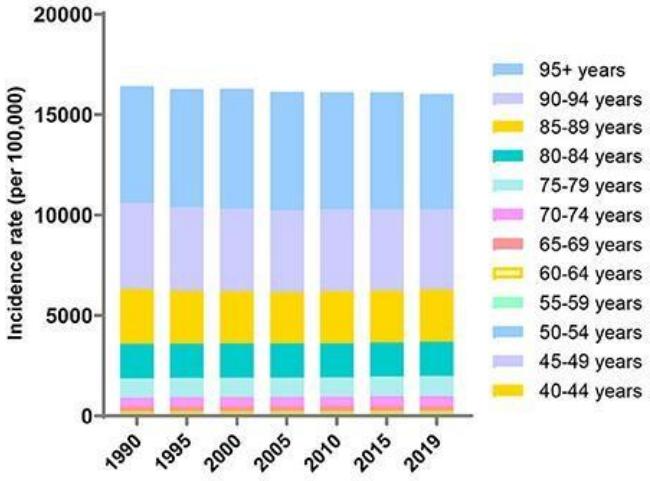


Figure 1: Illustration of the prevalence of Alzheimer's and other dementia cases by the age group in 204 countries and territories from 1990 to 2019*. (Li et al., 2022).*

1.2 Symptoms

Alzheimer's disease is recognised for its slow progression rate which often spans over thirty to forty years (Alzheimer's Association, 2022). The mild symptoms steadily develop into more severe health conditions, which often result in death at the latest stages of the disease (Tiwari, Atluri, Kaushik, Yndart, & Nair, 2019).

Early-stage Alzheimer's usually manifests with mild memory impairment and mood changes (Gottesman & Stern, 2019). The mild nature of the symptoms often makes it harder to spot the disease at its early stage. Individuals may report having difficulty remembering recent events and conversations or thinking of the right word in a certain context (Kazui et al., 2005). Patients are also known to become more depressed, irritated, or anxious (Robins Wahlin & Byrne, 2011).

Those diagnosed with the mild stage of the disease often continue to work, maintain healthy relationships, and participate in usual day-to-day activities despite requiring occasional support and guidance from those around them. However, Alzheimer's is a progressive neurodegenerative disorder, and people tend to lose their independence as the later stages of the disease approach (Scheltens et al., 2021). The physical capacity to complete daily tasks, such as personal care and feeding, is often lost, and personality changes become highly evident. These include, but are not limited to, social withdrawal, changes in sleeping habits, loss of inhibitions, and change of character (Terracciano & Sutin, 2019).

A compelling amount of research points to progressive morphological abnormalities of brain areas associated with memory, learning, and thinking, such as the hippocampus, entorhinal and perirhinal cortexes (DeTure & Dickson, 2019). A recent study narrowed down two brain areas which appear to be affected before any other: the hippocampus and amygdala (Planche et al., 2022) (Fig. 2).

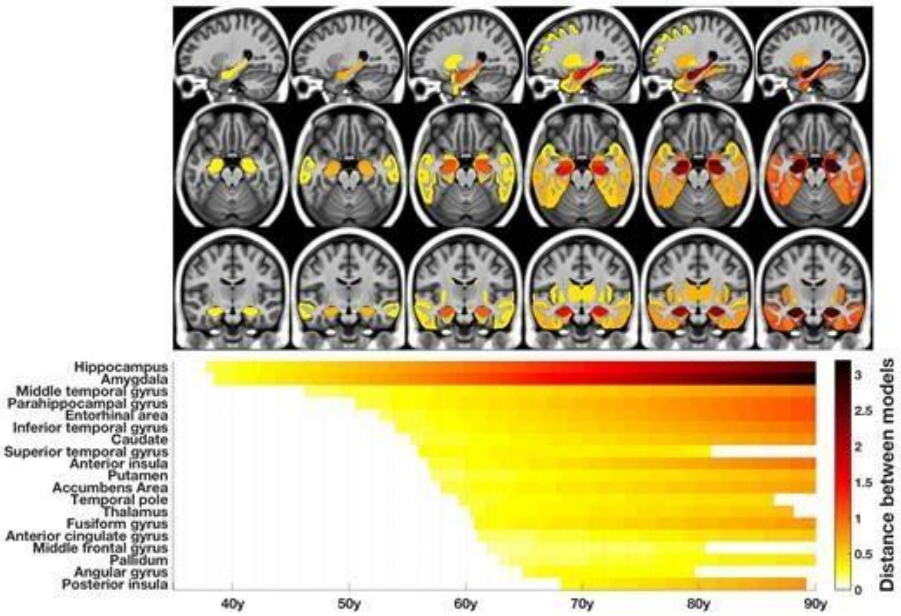


Figure 2: The chronological MRI staging of structure progression of Alzheimer's disease based on the distances of the disease trajectories*. (Planche et al., 2022)*

The research on mild Alzheimer's cases also demonstrated that amygdala atrophy is proportional to hippocampal atrophy (Poulin et al., 2011). These findings explain the fits of anxiety and irritability which often accompany the memory deficits at the earliest stages of the disease. In addition to the most common mental health issues, such as depression and anxiety, there are often other neuropsychiatric symptoms which can be a hallmark of the condition. Disinhibition, delusions, hallucinations, and aggression are also commonly present in those suffering from Alzheimer's (Cummings, 2000) (Fig. 3).

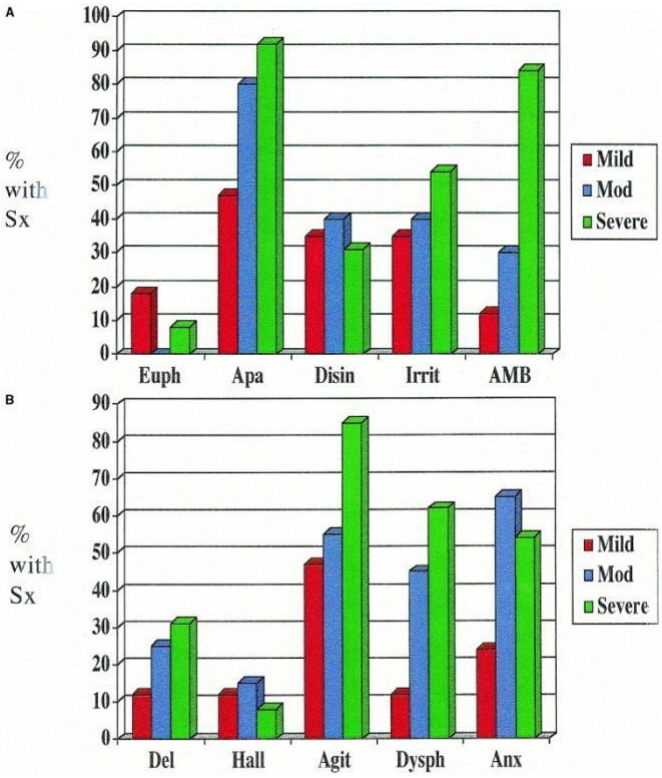


Figure 3: Manifestation of Neuropsychiatric symptoms in Alzheimer's cases*. Euph = euphoria; Apa = apathy; Disin = disinhibition; Irrit = irritability; AMB = aberrant motor behavior; Del = delusions; Hall = hallucinations; Agit = agitation; Dysph = dysphoria; Anx = anxiety; (Cummings, 2000)*.

As the disease progresses, other brain areas become affected, and new symptoms appear. People may find it increasingly harder to make decisions and address the issues with the former clarity of mind (El Haj et al., 2020). Lack of judgement is also becoming more evident as the patient's quality of life may start to suffer from poor decision-making (DeTure & Dickson, 2019). If a severe lack of mental capacity is recognised, carers and professionals may make decisions on behalf of that person about their property, health care, and finance. All these are covered by the governmental legislations (Mental Health Capacity Act, 2005).

Aphasia is another cognitive symptom that is widely recognised for its detrimental effect on the quality of life. The manifestation of language deficit may further aggravate the mental state of those suffering from the condition and contribute to the feeling of isolation (Srivastava, Ahmad, & Khare, 2021). Both the semantic and pragmatic levels of language processing are affected. Patients may have difficulty understanding the words and their meaning, maintaining language fluency, and appropriately adapting one's language to specific social contexts (Ferris & Farlow, 2013).

At the most advanced stages of Alzheimer's, the neurodegeneration reaches the brain regions responsible for the basic bodily functions, increasing the risk of death from the disease complications. A person is reported to have difficulty eating, swallowing,

walking, or fighting off infections (Scheltens et al., 2021). Alzheimer's is accompanied by an increased risk of cardiovascular diseases, pulmonary embolism, cachexia, dehydration, and falls (Todd, Barr, & Passmore, 2013). A systematic review and meta-analysis also concluded that dementia patients were two times more likely to have pneumonia as a cause of death in comparison to the age-matched controls without the dementia diagnosis (Manabe, Y., Mizukami, Akatsu, & Kudo, 2019).

1.3 Pathophysiology

The main pathophysiology of Alzheimer's disease is an extracellular accumulation of Amyloid β (Aβ) plaques, formed by an aggregation of misfolded amyloid-β proteins (Weller & Budson, 2018). An intraneuronal aggregation of hyperphosphorylated Tau forms neurofibrillary tangles (NFTs), which are the second major hallmark of Alzheimer's pathology (Weller & Budson, 2018). Tau is a microtubule (MT)-binding protein which requires phosphorylation of certain sites for successful binding to MTs. Upon formation, NFTs locate in the neuronal cytoplasm and disturb neuronal communication thus further contributing to cell loss (Menkes-Caspi et al., 2015).

The relationship between the Aβ and NFT deposits is a complex one and it is commonly believed that Aβ plaques precede NFTs. A review by (H. Zhang et al., 2021) provides a comprehensive overview of their association and influence on one another. Aβ fibrils appear to aggravate Tau hyperphosphorylation and its detachment from the MTs by upregulating GSK-3β and CDK-5 enzymes that are involved in Tau phosphorylation. Upon its detachment from the MTs, the affinity of the hyperphosphorylated Tau to other monomers increases, and new deposits form. However, the connection between Aβ and hyperphosphorylated Tau deposits is bidirectional. Although the influence that Tau has on Aβ is less clear, there is evidence linking the increased expression of Tau with Aβ-related neurotoxicity.

(Zhao, Liu, Xia, Zhang, & Wang, 2020) reviews the mechanism of amyloidogenic and nonamyloidogenic pathways which both begin with the amyloid precursor protein (APP) that misfolds into amyloid-β proteins. APP is an integral transmembrane protein with extracellular domains. APP can be cleaved by one of the three enzymes: β-secretase, γ-secretase, or α-secretase (Fig. 4). In the nonamyloidogenic pathway, APP is cleaved by the α- secretase at 16-17 residues to produce soluble precursors and a large soluble ectodomain. α-secretase generates a soluble N-terminal fragment (APPsα) and a membrane-bound C-terminal fragment, C83, which undergoes further

cleavage to make a soluble extracellular p3 peptide. APPsα acts as a neuroprotective factor and plays a role in cell-substrate adhesion. Its release is essential for normal synaptic signalling, plasticity, neuronal survival, and gene expression. In contrast, the activity of β- and γ-secretases present in the amyloidogenic pathway leading to the severe accumulation of Aβ. β-secretase acts first and cuts a large portion of the peptide to produce a soluble N-terminal fragment (APPsβ) and a membrane-bound C-terminal fragment (C99). γ-secretase then cleaves the C99 and generates a cytoplasmic polypeptide termed AICD, which travels into the nucleus where it acts as a transcription factor, and the Aβ peptides.

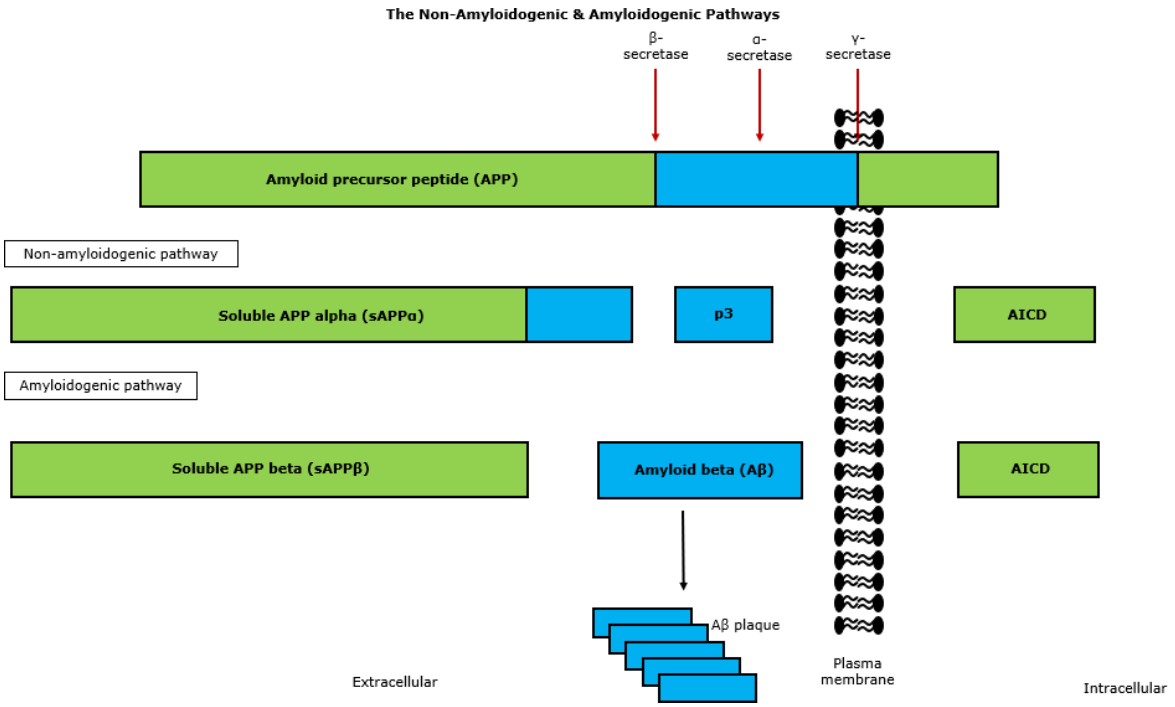


Figure 4: A simplified illustration of the non-amyloidogenic and amyloidogenic processing of APP*. APP can be processed by different secretases. α-secretase generate soluble APPα and p3 which are the hallmarks of physiological processes. The cleavage of β- and γ-secretases produces soluble APPβ, AICD, as well as Aβ proteins. The latter further aggregate to form Aβ plaques.*

There are different types of Aβ polymeric fibrils of different lengths, ranging from 38 to 43 residues (Pauwels et al., 2012). Different Aβ fibrils have different tendencies for self-aggregation. The formation of Aβ40/Aβ42 polymers is commonly found in early-onset Alzheimer's cases and is associated with reduced activity of the ion channels, disturbed calcium homeostasis and energy metabolism, and increased oxidative stress (Tiwari et al., 2019).

1.4 Diagnostics

The diagnostic criteria recognise three stages of Alzheimer's: 1) a preclinical stage with no symptoms but possible presence of subtle, disease-associated biological activity; 2) a middle stage of low to mild cognitive impairment; and 3) a severe stage with serious and life-threatening symptoms (Weller & Budson, 2018).

In the earlier years of Alzheimer's research, the diagnosis could only be confirmed after death (Jost & Grossberg, 1995) by performing post-mortem pathological brain examinations using donor brains (Braak & Braak, 1991). Modern clinicians now have a wide range of testing approaches. A unified approach is currently in use, known as the AT(N) Framework, which stands for amyloid plaques, Tau tangles and neurodegeneration in the brain (van der Flier & Scheltens, 2022). The presence of all three characteristics is used to diagnose and determine the stage of the pathology. Primarily, the biomarkers such as Aβ peptides and hyperphosphorylated Tau are quantified in the cerebral spinal fluid of the patients (Gonzalez-Ortiz et al., 2022). Physical and neurological exams targeting reflexes, coordination, balance, and muscle tone are likewise assessed (Zverova, 2019). Blood tests are also usually performed to rule out other potential causes of the symptoms, such as thyroid dysfunction or vitamin deficiency (Tiwari et al., 2019). Advanced brain imaging techniques are another efficient diagnostic tool that provides information about the brain metabolism (i.e. Fluorodeoxyglucose PET scan), presence of diseased deposits(i.e. Amyloid and Tau PET imaging), and functional abnormalities (i.e. MRI, Computerized Tomography). But despite their prolonged use, CSF sampling and neuroimaging techniques pose multiple financial, practical, and logistical issues (Gonzalez-Ortiz et al., 2022). Therefore, the development of AT(N) blood biomarkers would be a more convenient, accessible, and cost-effective tool, which can be used in diagnostic, prognostic, and therapeutic trial applications (Gonzalez-Ortiz et al., 2022). Instead of the conventional CSF sampling, plasma amyloid beta (Aβ)42/Aβ40 can be measured using immunoprecipitation-mass spectrometry methods. Plasma p-tau quantification has also been found to be effective at detecting the abnormalities characteristic of Alzheimer's pathology. Most importantly, both approaches are good at differentiating Alzheimer's disease from other neurodegenerative pathologies (Gonzalez-Ortiz et al., 2022).

Due to the low prevalence of familial Alzheimer's, genetic screening isn't a common

practice but does provide an almost certain guarantee of disease development for someone with familial mutations (Giau et al., 2019).

1.5 Genetic Predisposition

Alzheimer's disease is traditionally divided into late-onset (LOAD) and early-onset (EOAD), or sporadic and familial Alzheimer's disease. The sporadic nature of the disease accounts for the majority of cases with only 0.1% of all cases having a familial origin (Srivastava et al., 2021).

EOAD has an autosomal dominant inheritance and is linked to mutations in three genes: APP, PSEN1 and PSEN 2 (Latimer, Lucot, Keene, Cholerton, & Montine, 2021). Around 270 mutations in thesegenes are currently known to cause familial cases of Alzheimer's disease, and the new point mutations continue to be unmasked (Giau et al., 2019). Such familial casesmanifest before the age of 65 but follow the same progression patterns as sporadic cases, which develop later in life (Weller & Budson, 2018). Interestingly, both presenilin genes are part of the γ-secretase (Veugelen, Saito, Saido, Chavez-Gutierrez, & De Strooper, 2016). In addition to Aβ formation, mutations in the APP and PSEN1 genes impact pathways of the immune system, BBB, synaptic plasticity, axonal guidance, and apoptosis (Hoogmartens, Cacace, & Van Broeckhoven, 2021).

In contrast to EOAD, which is caused by highly penetrant mutations in a few genes, LOAD is associated with numerous low-penetrance genetic variants. The critical contributor to sporadic Alzheimer's is the APOE gene, which is estimated to account for ≈50% of LOAD phenotypes (Naj, Schellenberg, & Alzheimer's Disease Genetics, 2018). The other half can be explained by many other genes which have been associated with LOAD over the years: ACE, CHRNB2, CST3, R1, GAPDHS, IDE, MTHFR, NCSTN, PRNP, PSEN1, TF, TFAM and TNF (Naj et al., 2018).

APOE is located on chromosome 19 (19q13.2), where it codes for the production of the Apolipoprotein E (ApoE), a protein involved in lipid transport, storage, and metabolism (Tiwari et al., 2019). There are three known alleles of the gene that code three different protein isoforms: ApoE2, ApoE3, and ApoE4. APOE2 allele is relatively rare, and it provides a certain protection against the disease (Shinohara et al., 2020). APOE3 is believed to have a neutral role in the onset of the Alzheimer's (Frieden, Wang, & Ho, 2017). APOE4 is the allele that is regarded as a strong risk for LOAD

(Shinohara et al., 2020).

Mutations associated with LOAD, including APOE4, are low penetrant. Therefore, instead of causing the disease, they simply increase the risk, which can then be influenced by lifestyle habits and environmental factors (Tiwari et al., 2019). Furthermore, the risk of Alzheimer's development seems to be greatly increased by the conditions that affect the heart, blood vessels, and the gut – heart disease, obesity, diabetes, stroke, high blood pressure, gut dysbiosis, and high cholesterol are the conditions that increase the odds of developing the pathology (Weller & Budson, 2018).

However, there is a growing concern that the separation of Alzheimer's cases into familial and sporadic onset is not as clear as we believe. Therefore, a more precise separation of EOAD cases has been proposed: mendelian (mEOAD), and nonmendelian (nmEOAD), both of which have been described by (Reitz, Rogaeva, & Beecham, 2020).mEAOD is what we usually understand as a familial type of the disease – these havean autosomal dominant inheritance pattern with fully penetrant genes (i.e. APP, PSEN1, PSEN2). But despite the common understanding that this is what constitutesfamilial Alzheimer's, only 10% of EOAD cases are accounted for by mutations in thesegenes. Therefore, an alternative explanation must be drawn about the nature of the EOAD. It was therefore proposed that nmEOAD manifests with certain inconsistent inheritance patterns and that genetic factors are of multifactorial and polygenic nature.There is still a lack of understanding of what the clinical and etiological distinction is between nmEAOD and LOAD. One of the theories is that nmEOAD is a severe phenotype of LOAD and develops out of accumulated LOAD-associated genetic factors and possibly other genetic-environmental factors which lower the onset of the disease. There are over 30 loci that can modify disease susceptibility by acting on molecular pathways associated, such as the immune response and lipid metabolism.Despite the obvious advances of the last decade in this area, a clear understanding is still missing of the neuropathologic delineation of mEOAD from nmEOAD.

2. The Gut-Brain Axis and Role of Diet in Alzheimer's Disease

Dietary habits have been brought to the spotlight due to the interest in the connection between the microbiota-gut-brain axis and neurodegenerative diseases. The Gut-

Brain-Axis (GBA) is a bidirectional communication link between the enteric nervous system and the central nervous system (CNS) (Fig. 5). It comprises metabolic, immunological, endocrine, Vagus nerve signalling and microbial metabolites, such as short-chain fatty acids (SCFAFs), proteins, and tryptophan metabolites (Miller, Bessho, Grando, & Tukel, 2021). This communication system regulates essential physiological and homeostaticfunctions which include food intake, immune and endocrine system regulation, sleep,and mood (Cryan et al., 2019).

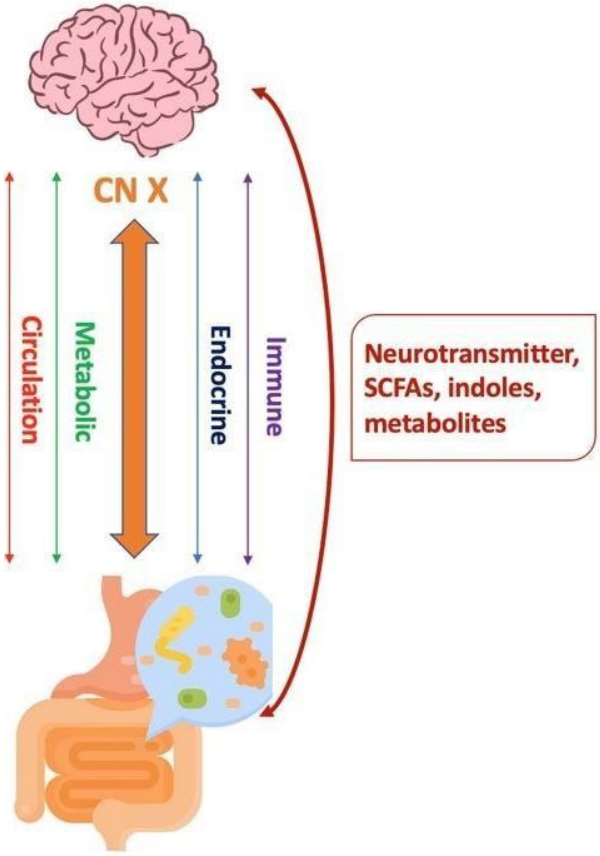


Figure 5: The overview of the bidirectional connection between the brain and the gut*. There are multiple modes of communication between the gut and the brain with each being impacted in the neurodegenerative diseases.*

The gut microbiota has emerged as a particularly important factor that determines the function of the GBA – it is a complex, symbiotic environment that comprises more than 100 trillion symbiotic cells and hosts various microbes, such as bacteria, archaea, viruses, and fungi (Doifode et al., 2021). Healthy microbial composition ensures there is an adequate synchronization between the gut and the central nervous system and is linked to healthy ageing (Ragonnaud & Biragyn, 2021; Wilmanski et al., 2021). 95%of microbiota that colonises a human body is localised in the gut where it forms a reciprocal relationship with its human host and plays a direct role in protecting the host against pathogens (Cryan et al., 2019). Microbiota carries out its protective functioning by carrying out crosstalk with the mucosal immune system, integrating signalling pathways and gene regulation (Miller et al., 2021). One of the most influential pathways, through

which diet can facilitate neurodegeneration, is by causing dysbiosis and inflammatory response (Kincaid, Nagpal, & Yadav, 2021; Martin, Osadchiy, Kalani, & Mayer, 2018) (Fig. 6).

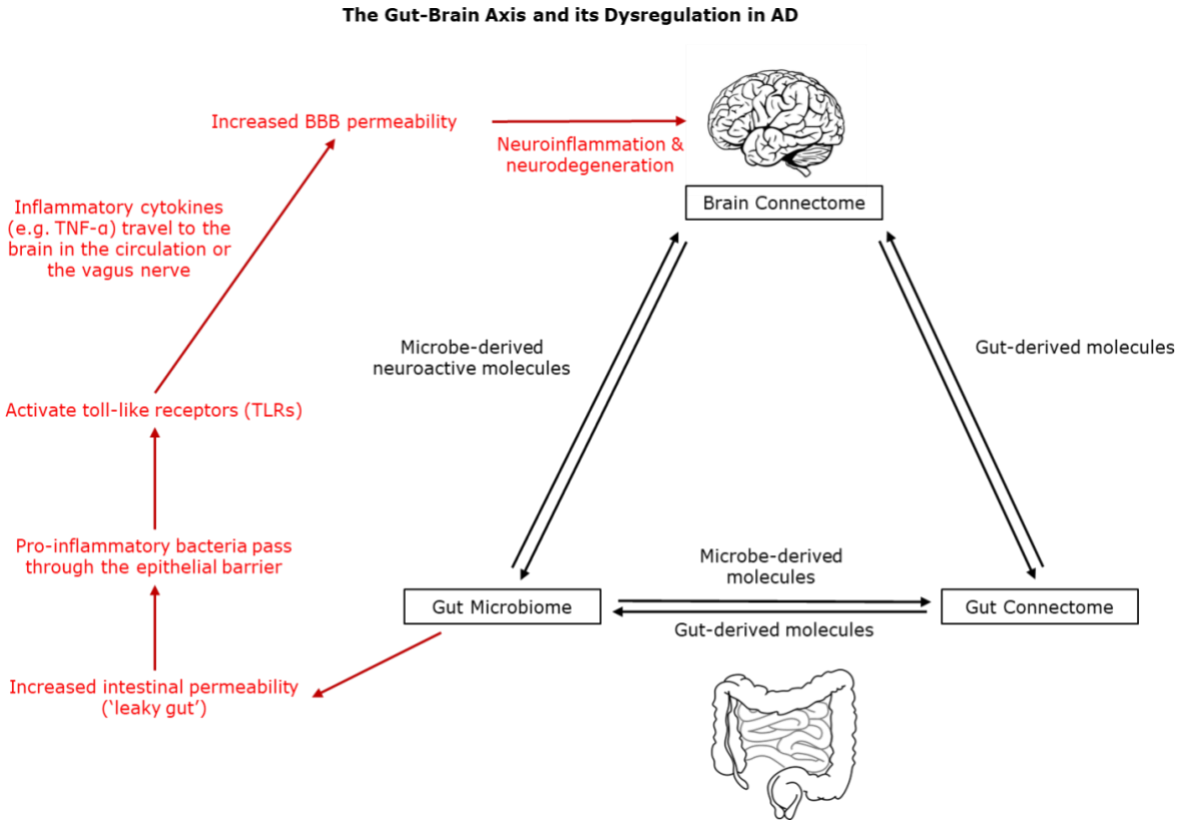


Figure 6: A simplified illustration to represent the role of the GBA in facilitating inflammatory response and neurodegeneration*. By acting on the microbiome composition and its permeability, diet can increase the expression of pro-inflammatory bacteria which will lead to a cascade of inflammatory responses. Resulting damage to the Blood-brain-barrier integrity is one of the events which accompany the early stages of Alzheimer's as it can further increase inflammation and neurodegeneration in the brain (Martin et al., 2018).*

As the interest in nutrition and its role in cognitive performance started to grow, numerous studies sought to investigate the effects of different foods on brain health. Consumption of fruits and vegetables has been associated with cognitive health (Zhou Y. et al., 2022) and slower cognitive ageing (Morris et al., 2018). Whereas components found in seafood, such as omega-3 fatty acids and monounsaturated fatty acids, have been associated with anti-inflammatory and neuroprotective effects (Fotuhi, Mohassel, & Yaffe, 2009; Panza et al., 2004). Other foods like whole grains (Dalile et al., 2022; Leidman, Doocy, Bollemeijer, Jatoi, & Majer, 2022), olive oil (Roman, Jackson, Gadhia, Roman, & Reis, 2019), nuts (Ros & Sala-Vila, 2020), and legumes (Mazza et al., 2017), have also been linked with better global cognitive performance. These foods

are common in the Mediterranean Diet, which is known as an abundant source of vitamins, antioxidants, minerals, and healthy fats (Aridi, Walker, & Wright, 2017). Not surprisingly, a growing body of evidence links the Mediterranean Diet with healthy ageing and better cognitive performance (Ballarini et al., 2021; Feart, Samieri, & Barberger-Gateau, 2010; Morris et al., 2018; Scarmeas, Anastasiou, & Yannakoulia, 2018). In contrast, the Western Diet is increasingly associated with Alzheimer's disease development and greater cognitive impairment (Lopez-Taboada, Gonzalez-Pardo, & Conejo, 2020). As opposed to the Mediterranean Diet, the Western Diet is highin sugars and saturated fatty acids, with very low amounts of fibre and antioxidants intake (Doifode et al., 2021). Chronic exposure to such dietary habits is known to increase metabolic and oxidative stress markers expression (Morris et al., 2018). It was also previously shown that high-fat diet could affect cholesterol biosynthesis and metabolism by acting on Na+/K+-ATPase signalling pathway (L. Wang, Xu, Zhang, Jin, & Li, 2015) and HMG-CoA reductase (Spagnuolo et al., 2020), and overall rate of cholesterol synthesis (Quan, Xie, Dietschy, & Turley, 2003). Diet-induced inflammatory responses and BBB damagewere shown to be paralleled by the accumulation of toxic fibrils (Wieckowska-Gacek, Mietelska-Porowska, Wydrych, & Wojda, 2021). Taken together, this evidence highlights the important role that the Western Diet has in driving neurodegenerative events and Aβ plaque formation.

3. Cholesterol-Induced Toxicity in Aβ accumulation

Cholesterol is a highly hydrophobic molecule found in cellular membranes, where it controls the membrane fluidity and trafficking, lipid cluster formation, the affinity of proteins to lipid clusters, and signal transduction (Luo, Yang, & Song, 2020). Other cholesterol functions involve the mediation of neuronal excitation, synaptic transmission, and evenneuronal tissue morphology (Dietschy & Turley, 2004). The brain is thus the most cholesterol-rich organ and contains as much as 25% of non-esterified cholesterol (Dietschy & Turley, 2001).

Brain cholesterol is essential for myelin membrane growth (Saher et al., 2005), with the myelin containing up to 70% of the whole brain cholesterol pool (Russell, Halford, Ramirez, Shah, & Kotti, 2009). Due to its strong role in myelination, cholesterol synthesis is observed both in neurons and astrocytes during embryogenesis and early childhood (Czuba, Steliga, Lietzau, & Kowianski, 2017). Upon completing their

differentiation, adult neurons give up their ability to produce cholesterol and the astrocytes become the primary site of cholesterol biosynthesis and export (Nieweg, H., & Pfrieger, 2009). Upon production in the astrocytes, cholesterol mediates membrane fluidity, lipid raft formation, and carbohydrate metabolism (Giau et al., 2019). The astrocyte-based cholesterol synthesis is low

during embryonic development but becomes high shortly after birth (Nieweg et al., 2009). In contrast, neuron-synthesised cholesterol is detected in high concentration during embryonic development and then reduces to negligible amounts during adulthood (Nieweg et al., 2009). Other glial cells, such as oligodendrocytes and microglia, also demand astrocyte-derived cholesterol and drive the cholesterol function in phagocytosis, immune surveying, and synapse pruning (Nieweg et al., 2009).

The production of brain cholesterol is distinct from the synthesis process in the rest of the body. In the periphery, 70% of cholesterol undergoes *de novo* biosynthesis in the liver, while 30% comes from the diet (Qian, Chai, Gelissen, & Brown, 2022). The cholesterol is then transported to the rest of the body via lipoprotein-mediated lipid transfer (J. Zhang & Liu, 2015). However, the BBB provides a reliable obstruction to the cholesterol originating elsewhere in the body (Genaro-Mattos, Anderson, Allen, Korade, & Mirnics, 2019). Under healthy conditions, the brain remains free of dietary cholesterol and the brain cholesterol levelsare maintained by *de novo* synthesis (J. Zhang & Liu, 2015). This is a resource- expensive process as 23 enzymes are involved in the conversion of the precursor Acyl-coenzyme A (CoA) into the final sterol. In the event when cholesterol levels are high, its excess amounts are transported from the plasma membrane to the ER, where it inhibits SREBP2, a rate-limiting enzyme necessary for cholesterol synthesis, thus preventing further cholesterol production. In addition, ER-bound transcription factor Nrf1 initiates cholesterol extrusion (Widenmaier et al., 2017). Cholesterol synthesis and maintenance are therefore two very tightly regulated processes, which are performed successfully under healthy brain conditions. However, pathological conditions, which result from ageing or disease, often disturb this fine homeostasis andcause a variety of harmful metabolic reactions. This supports the strong connection between the disruption of cholesterol homeostasis and various disease including Alzheimer's.

One of the primary functions of cholesterol in the neurons is to regulate the affinity of proteins to the lipid clusters. APP is one of the proteins whose affinity to lipid clusters cholesterol is known to regulate, initiating either the amyloidogenic or non-amyloidogenic pathways. β- and γ-secretases are believed to locate in the lipid clusters in contrast to α-secretases, which reside outside the lipid clusters in a region

of disordered polyunsaturated lipids (Yang, Sun, Eckert, & Lee, 2014). A recent study employed a super-resolution imaging technique to observe the APP trafficking in and out of the lipid cluster (H. Wang et al., 2021). They showed that cholesterol-rich ApoE drives theAPP towards the β- and γ-secretases in the lipid clusters to generate Aβ fibrils. In contrast, ApoE carrying low cholesterol concentrations was shown to move the APP from the lipid clusters to associate with α-secretase thus leading the APP down the non-amyloidogenic pathway Interestingly, the ε4 allele of ApoE is strongly associated with the onset of Alzheimer's disease as well as higher brain cholesterol levels (Kojro, Gimpl, Lammich, Marz, & Fahrenholz, 2001). Both cleavage pathways appear to be regulated by cholesterol, but the difference seems to result from the concentration of cholesterol present in the cellularenvironment (Fig. 7).

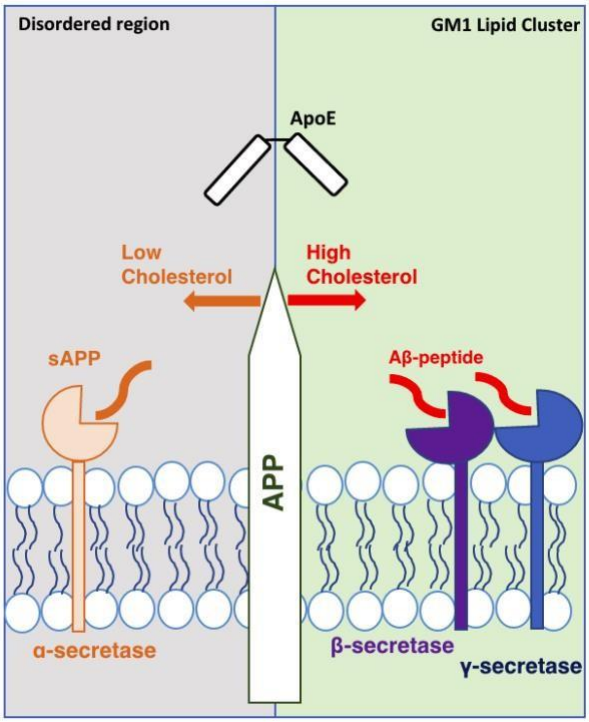


Figure 7: Cholesterol-mediated APP trafficking in and out of the lipid cluster towards b- & y- secretases or a-secretase*. ApoE-regulated high cholesterol concentration results in translocation of APP into lipid clusters where it follows amyloidogenic pathway. Under low cholesterol levels, APP dissociates from GM1 and is cleaved by α--secretase. Adapted from Wang et al., 2021.*

4. The Role of the Blood-Brain-Barrier in the Cholesterol-Induced Toxicity

The BBB is a highly specialised system with several essential functions in the CNS including the regulation of cerebral flow, and the transport of oxygen and beneficial nutrients, such as glucose (Sweeney, Sagare, & Zlokovic, 2018). The barrier also acts as a shield,preventing the entry of toxic substances from the blood and removing harmfulcompounds from the brain (Michalicova, Majerova, & Kovac, 2020). The BBB is an important part ofthe neurovascular unit which also contains neurons, astrocytes,

myocytes and

pericytes and regulates the structural and functional relationship between the brain and blood vessels (Kadry, Noorani, & Cucullo, 2020).

A monolayer of brain microvascular endothelial cells (BMVEC) is one of the primary structural components of the BBB, and their functions include transport of micro- and macro-nutrients, osmoregulation, and receptor-mediated trafficking (Huang, Hussain, & Chang, 2021). BMVEC contains a larger quantity of mitochondria than the endothelial lining ofother organs, which enables the active transport of nutrients across the membrane (Persidsky, Ramirez, Haorah, & Kanmogne, 2006). The tight junctions between endothelial cells are the main characteristic of the barrier as they create a physical obstacle crucial to the functions of the BBB. The outer layer of the endothelial lining is covered by pericytes and the end-feet of astrocytes, which are reported to cover most of the BMVEC surface area (Huang et al., 2021).

The BBB plays a major role in protecting the fine balance of cholesterol homeostasis in the brain and prevents the passage of lipoprotein-bound cholesterol from the circulation, ensuring that *de novo* synthesis is the only major source of cholesterol in the brain (Sweeney et al., 2018). It has been suggested that the reason for this set-up lies in the need for constant cholesterol expression in the membranes and myelin. Therefore, it would have been difficult to maintain such constant levels of cholesterol if it had to be constantly transported in the form of lipoprotein from the circulation (Saeed et al., 2014).

Even though the brain cholesterol pool is mostly separated from the general circulation, some cholesterol oxysterol metabolites can be exchanged between the body and the brain: 7-hydroxycholesterol (27-OHC) and 24S–hydroxycholesterol (24S–OHC) (Czuba et al., 2017). Both side-chain hydroxylated metabolites are good inhibitors of cholesterol biosynthesis. It was reported that about 66% of cholesterol production is balanced out by the generation of 24S-OHC which then diffuses out of the brain into the circulation (Czuba et al., 2017). In turn, 27-OHC can be absorbed by the brain and converted into a steroid acid, which also eventually leaves the brain and returns to the bloodstream (Russell et al., 2009). The disruption of either of these mechanisms can disturb the cholesterol exclusion of the brain, thus linking these oxysterols to neurodegeneration (Saeed et al., 2014).

Vascular pathophysiology, which results in a damaged BBB, is common in

neurodegenerative diseases including Alzheimer's (Zipser et al., 2007). One of the ways in which permeable BBB aggravates this condition is by allowing foreign and potentially harmful and inflammatory compounds into the brain (Sweeney et al., 2018). Secondly, structural alteration of the BBB leads to its inability to remove the toxic compounds from the brain environment (Sweeney et al., 2018). Taken together, these processes aggravate the toxicity and inflammation in the brain, creating perfect conditions for the further progression of neurodegeneration (Persidsky et al., 2006).

The hallmarks of Alzheimer's-related BBB pathology include abnormal permeability, microbleeds, loss of tight junctions and degeneration of endothelial and perivascular linings (Huang et al., 2021). In Alzheimer's disease, perivascular Tau aggregation and the accumulation of Aβ42 around the cerebral vessels can directly damage the BBB integrity, contributing to further inflammation and toxicity (Michalicova et al., 2020). The clearance mechanism, which exists to remove the protein aggregates from the brain, also loses its efficiency, which worsens the problem of protein aggregation (Huang et al., 2021). However, there is also evidence linking the BBB to the onset of the disease in addition to its role in the disease progression (Sweeney et al., 2018) as the new evidence suggests that mild to moderate damage to the BBB integrity is also a part of healthy ageing (Erickson & Banks, 2019).

We are interested in the role that the BBB plays in the onset of the disease and how hypercholesterolemia may contribute to this pathology. Recent compelling research points to the role of metabolic overload in damaging the BBB integrity, even in the absence of the active stage of disease (Deane & Zlokovic, 2007; Sheikh et al., 2022; Zipser et al., 2007). Similarly, BBB disruption emerged as a critical event in Alzheimer's brain alteration following diet-induced high cholesterol levels (de Oliveira et al., 2020) and some studies have investigated potential pharmaceutical intervention in attenuating cholesterol-mediated BBB breakage (Jiang, Xia, Jiang, Wang, & Gao, 2014). Cholesterol was also shown to enter the brain from the open circulation in mutants with increased BBB permeability and this flux upregulated brain cholesterol biosynthesis even more (Saeed et al., 2014).

5. The Role of Oxidative Stress in Alzheimer's Disease Pathology

Oxidative stress also links to increased inflammation in the brain and becomes implicated in many cognitive deficit pathologies (Tan & Norhaizan, 2019). It results

from the excess presence of free radical molecules, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS), which are highly unstable and reactive, causing substantial damage to the neuronal tissue (Cassidy et al., 2020). In moderate concentrations, free radicals are a normal product of cellular metabolic processes (Sies, Berndt, & Jones, 2017). However, under pathological condition, free radicals' production becomes so excessive, that it outweighs the capabilities of the antioxidant system (Pena-Bautista et al., 2019).

There are several major ways in which oxidative stress is associated with the severity of neurodegeneration. Primarily, metal ion homeostasis is disrupted under oxidative stress conditions, which prevents metal ions from healthy participation in biological functions (Sies et al., 2017). Metal ion disruption may also cause further upregulation of ROS, resulting in imbalance of phosphatases and secretases function and acceleration of Aβ and NFTs aggregation (Birla, Minocha, Kumar, Misra, & Singh, 2020).

Oxidative stress also leads to macromolecule peroxidation which is associated with accelerated ageing and multiple chronic diseases (Cassidy et al., 2020). The phospholipid components of cellular membranes are a major target of oxidative stress attacks due to the high lipid composition in the brain and its high oxygen demand (Ferre-Gonzalez, Pena-Bautista, Baquero, & Chafer-Pericas, 2022) as the increased risk of free radical production is a potential side-effect of high oxygen consumption (Banks & Rhea, 2021). This event contributes to the formation of Aβ plaques and deposits of hyper-phosphorylated Tau proteins (Steinert & Amal, 2023) since lipid peroxidation directly damages neuronal membranes and causes free radical-mediated injury of cellular components (Lehner et al., 2011; Sultana, Perluigi, & Butterfield, 2013). Oxidative attacks on the polyunsaturated fatty acidsresult in their intramolecular reorganisation and change to their protein structure (Ito, Sono, & Ito, 2019). These structural changes have been linked to covalent modification of the proteins (Grimsrud, Xie, Griffin, & Bernlohr, 2008), inflammation (Sottero, Rossin, Poli, & Biasi, 2018), toxicity (Obulesu, Venu, & Somashekhar, 2011), and cell death (Iuchi, Takai, & Hisatomi, 2021). Such alterations have also been reported at the very early stages of Alzheimer's disease (Montine et al., 2002), suggesting that this process is strongly implicated in the initial onset as well as the progression of the disease.

The BBB was also shown to suffer from oxidative damage attacks, damaging the

BMVECs, pericytes and astrocytes and destroying the molecular composition of the BBB (Montine et al., 2002 (Song & Li, 2020). Diets high in saturated fats, cholesterol, sugar, and salt contribute to oxidative stress levels and lipid peroxidation (Bojková, Kurhaluk, & Winklewski, 2021). Even though diet-derived saturated fatty acids are themselves not affected by peroxidation, they facilitate cellular oxidative stress by triggering downstream signalling pathways (Onyango, 2021).

Similar to vertebrates, insects possess the BBB which protects their brain from potentially harmful substances that circulate the body. The main distinction is that Drosophila does not have blood vessels but a vascular system with an open circulation which transports the haemolymph around the body (Limmer, Weiler, Volkenhoff, Babatz, & Klambt, 2014). One of the main ways in which Drosophila BBB protects the brain is by preventing the high dosages of K+, which is found in the haemolymph, from entering and damaging the brain (Hindle & Bainton, 2014). Due to the absence of blood vessels in the Drosophilavascular system, the BBB covers the outside of the brain and is composed of two layers of cells (perineurial glial (PG) cells and the subperineurial glial cells (SPG) (Limmer et al., 2014).

6. Research Question and Objectives

In the present research, we are studying a model that explains one of the mechanisms by which the high-fat diet may influence early neurodegenerative processes in the brain. The primary components of the model are 1) cholesterol content in the brain; 2) integrity of the BBB; 3) and the levels of lipid peroxidation.

The balance of cholesterol biosynthesis and metabolism is strictly controlled. Cholesterol is essential to normal brain function and its expression is closely monitored by an internal homeostasis apparatus. We introduced compelling evidence from previous studies showing that under physiological conditions, ApoE-transported cholesterol drives the APP out of the lipid clusters to be cleaved by α-secretase. In the case of pathologically high cholesterol levels, APP is pushed towards the β- and γ-secretases, thus switching the physiologically healthy process into an amyloidogenic pathway. We argue that the Western-like diet may be one of major causes of increased cholesterol levels in the brain, thus contributing to the conditions that favour the amyloidogenic pathway. It was previously shown that the high-fat diet could affect cholesterol biosynthesis and metabolism by acting on the signalling pathways involved

in those events. However, the high-fat diet could also increase the brain cholesterol pool by acting on the gut microbiota and initiating the inflammatory cascade which would result in BBB damage. Under healthy conditions, the brain is well protected by the BBB which prevents the entry of substances from the circulation. However, early neurovegetative events such as gut dysbiosis, inflammation, and oxidative stress, initiate the loss of the BBB integrity. Increased BBB permeability could hence allow the entry of bodily cholesterol which wouldn't be otherwise permitted to cross the barrier. The present research focused on one of such early neurodegenerative events which contribute to BBB breakage – lipid peroxidation. Figure 8 summarises all the components of the model and explains the relationship between them.

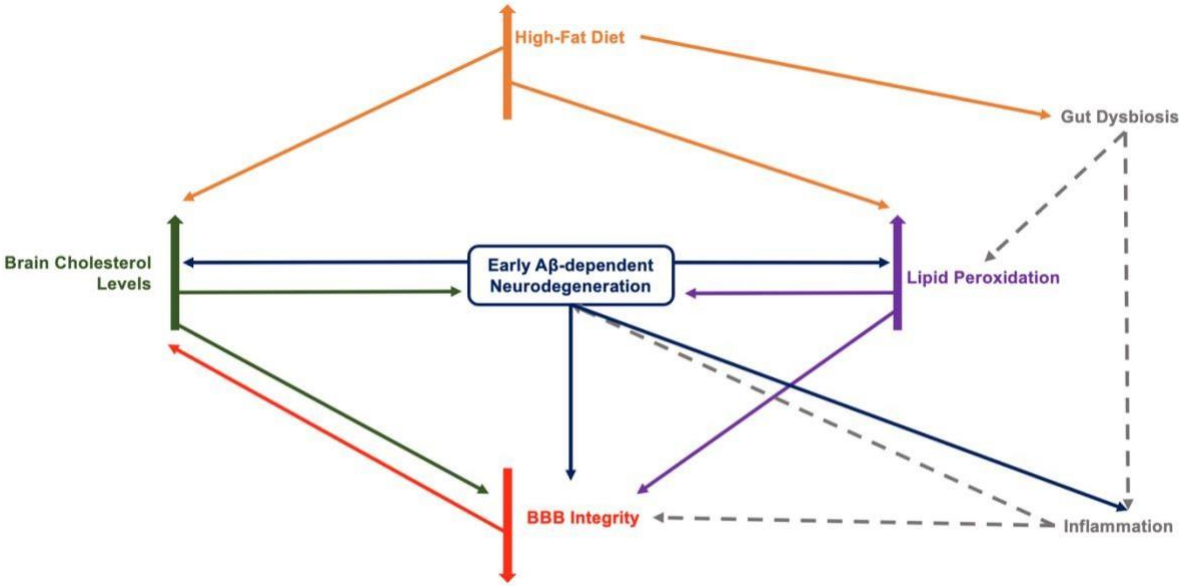


Figure 8: A simplified model depicting the mechanism by which high-fat diet may aggravate Aβ plaques pathology though gut dysbiosis, inflammation, hypercholesterolaemia, damaged BBB, and increased lipid peroxidation in the brain*. High-Fat diet acts on the GBA to induce inflammation and oxidative stress and undermines the BBB integrity. Increased permeability of the BBB allows circulatory cholesterol to enter the brain where it can disturb the fine balance of cholesterol synthesis and metabolism and facilitate Aβ fibrils aggregation. Neurodegeneration then further increases BBB permeability, inflammation, and oxidative stress.*

One of the most common reasons for failure in drug discovery is the lack of efficacy which often varies in the patient population (Fryburg, Song, & de Graaf, 2011). Therefore, it is believed that population stratification helps produce personalised and patient-specific therapeutic approach which increases the efficacy of a particular treatment (Hall, 2013). Therefore, we also aimed to examine the effects of the high-fat diet in multiple genetic populations to provide a comprehensive overview of the molecular mechanisms in different genetic groups. We hoped that results would help

us understand whether any genetic-related differences exist in the effects that the high-fat has on the disease progression. We prioritised two major mutations which correspond to familiar and the sporadic Alzheimer's risk mutations: $A\beta_{42}$ and hAPOE4 mutations, respectively. Finally, we aimed to develop and optimise experimental protocols which would allow us to investigate this model in the high-fat diet context in Drosophila Melanogaster (Fig. 9).

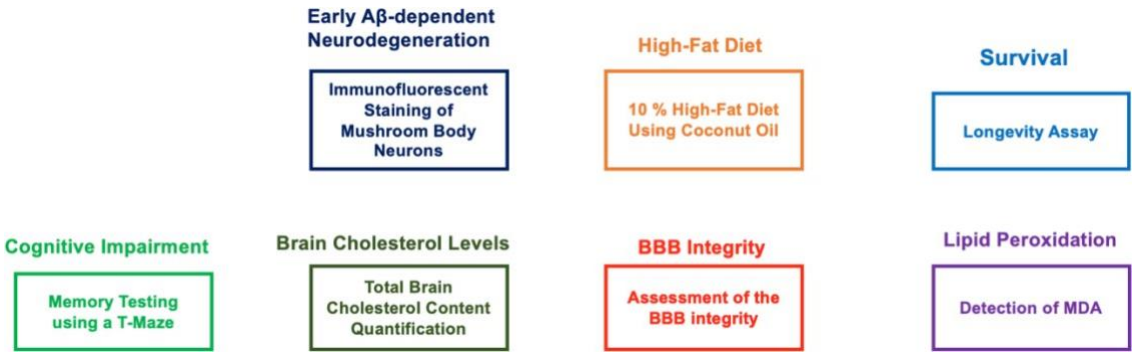


Figure 9: Overview of all the experimental protocols developed and optimised in this study.

Drosophila has long been used a model organism in disease research due to its short life span, convenient maintenance and genetic background that contains homologs for up to 75% human disease-causing genes (Mirzoyan et al., 2019). Drosophila is a powerful experimental model for studying Alzheimer's, which can be achieved by employing orthologs of human Alzheimer's -causing genes, transgenic constructs with disease allele and various models which allow us to study the effect of environmental factors (Bolus, Crocker, Boekhoff-Falk, & Chtarbanova, 2020).

GBA plays a pivotal role in our model of high-fat diet-induced neurodegeneration. Drosophila is a popular model in GBA research as the bacteria can colonise the fly gut, form fly-microbe symbiosis, and mimic human microbiome complexity (Ludington & Ja, 2020). Furthermore, Drosophila has previously helped elucidate the connections between microbiota, longevity, ageing and obesity in different disease models, including neurodegeneration (Kitani-Morii, Friedland, Yoshida, & Mizuno, 2021; Kong, Wang, & Jiang 2021; Sadaqat, 2021). The fly microbiota can be very easily manipulated as they can be made either sterile or mimicking standard human microbial composition (Douglas, 2018).

In the context of the BBB research, vertebrates and invertebrates have different origins and cellular composition of BBB (Dunton, Gopel, Ho, & Burggren, 2021). However, the

anatomic differences do not prevent the function of the BBB from being largely conserved across species, making Drosophila a reliable model for testing BBB permeability in different disease contexts (Contreras & Klambt, 2023).

Methods

Drosophila Melanogaster Husbandry

All flies were kept in controlled conditions with a 12 h:12 h light-dark cycle at 18 °C. All stocks were procured from procured from the Bloomington Drosophila Stock Centre. The following mutants were used in the study:

w^{1118}*:* Carries a mutation in the W gene which encodes an important protein in the eye pigmentation pathway.
UAS-hApoE4: Expresses the human APOE4 allele of the APOE gene under the control of UAS.
UAS-$A\beta_{42}$*arc*: UAS sequence drives the expression of an $A\beta_{42}$ peptide which carries the Arctic mutation with amino acid replacement E22G.

Genetic crosses were performed using Gal4/UAS system. 10-15 virgins of the elav-Gal4 driver line and young males of the UAS transgenes (Aβ; hApoE4)-containing line were collected and crossed. An egg-laying window was two days, after which the parent flies were removed from the vial. Offspring flies of our targeted mutation would hatch within 10 days after the egg-layingwindow was closed.

Males were collected for the experiments and female flies were disposed of. The research has initially began using both sexes but due to the time constraints and to prevent the sex from being one of the experimental conditions, only males were used in the experiments. We are aware of the limitations that arise from using only male flies as opposed to investigating both sexes. We recommend that all future research of this topic focuses on both males and females to account for any genetic and physiological differences.

At 3 days of age (*d.o.)*, flies were either put into standard (Fig. 10A) or high-fat food (Fig. 10B). Collection of flies for the assays was at the age of 8 and 23 days. The flies will continue to be classified as either 8 or 23 days old which would correspond to 5 and 20 days in high fat, respectively.

On the indicated collection date, all flies were anesthetised with CO_2 and prepared for either immunohistochemistry staining or biochemical experiments. The biochemical assay preparation involved the removal of heads and their subsequent homogenisation. Both the heads and the supernatant were stored at -80^{O}C until the day of the experiment. In contrast, confocal imaging required freshly dissected brains. Therefore, the immunohistochemistry protocol would begin on the same as the collection date and take around a week to complete.

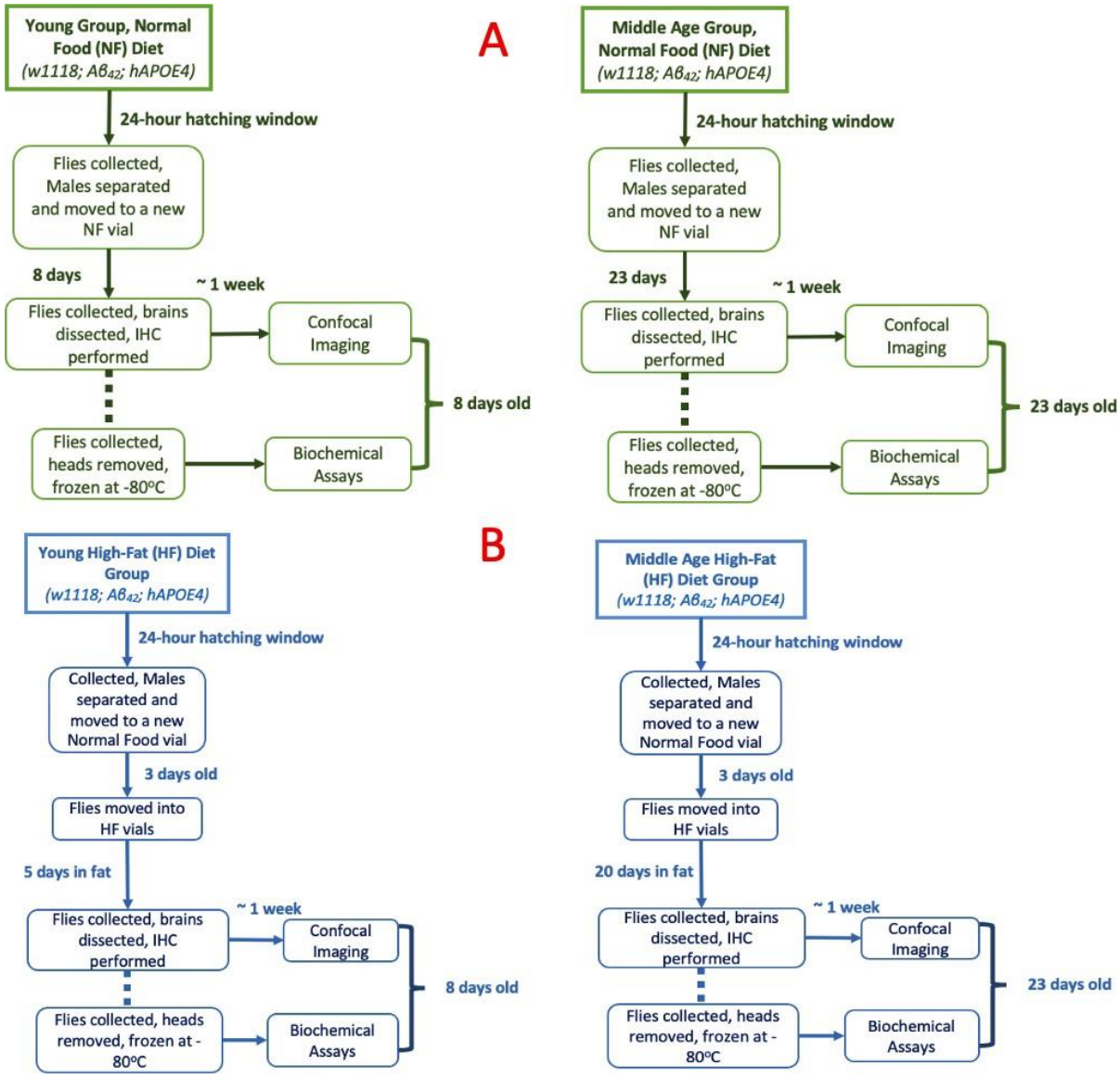


Figure 10: A timeline of Drosophila preparation for experiments. (A) *Drosophila lines on normal food.* ***(B)*** *Drosophila lines on high-fat food; IHC – immunohistochemistry.*

Flies of each experimental condition were kept in separate vials and colour coded. There were 41 vials in total, allowing accurate handling and preparation of flies for each experiment. Table 1 summarises the genetic lines and the number of flies used for each experiment.

		Immunofluorescent staining and confocal imaging		**Cholesterol Quantification Assay**		**Lipid Peroxidation Assay**		**Longevity**	
		Normal Food	High-Fat Diet	Normal Food	High-Fat Diet	Normal Food	High-Fat Diet	Normal Food	High-Fat Diet
Controls	w^{1118}	3	3	8	8	8	8	-	26

	8 days old								
	w^{1118} 23 days old	3	3	8	8	8	8		
EOAD	$A\beta_{42}arc$ 8 days old	3	3	8	8	8	8	25	22
	$A\beta_{42}arc$ 23 days old	3	3	8	8	8	8		
LOAD	hAPOE4 8 days old	3	3	8	8	8	8	28	25
	hAPOE4 8 days old	3	3	8	8	8	8		

Table 1: Summary of the Drosophila genetics lines, experimental conditions, biochemical experiments, and quantity of the male flies used in each experiment. *EOA: Early-onset Alzheimer's disease. LOA: Late-onset Alzheimer's. Heads were used for the biochemical assays and brains for the immunofluorescent staining and confocal imaging.*

Dietary Conditions Preparation and Handling

Normal Food (NF) consists of Cornmeal (536 g), Agar (42 g), Yeast (127 g), Soya flour (73 g), Sugar syrup (565 ml), Propionic Acid (35.4 ml), 20% nipagin in etoh (95 ml to 1 l), Methyl 4-hydroxybenzoate Bioreagent, Water. The food was prepared by a different Drosophila laboratory every two weeks at stored in a specifically designed storage area.

High-Fat food was prepared by mixing standard food with 10% organic coconut oil. Freshly prepared normal food was kept hot in the oven in a beaker until it was ready to be mixed with virgin coconut oil which was procured from a supermarket.

Rearing problems in high-fat diets: occasionally, a thin layer of melted coconut oil would remain on the top of the food. This is considered unfavourable as it signifies that the mixture was not mixed properly which would increase the overall stickiness of the food. To avoid such a separation of the oil and food, it was important to mix the content of the beaker very well. A magnetic stirrer was used to mix the coconut oil with the normal food for around 5 minutes. A mixture was then poured into vials which were

allowed to cool down before being put away in a 4ºC fridge for storage. To maintain the flies in the fresh high-fat food, it was prepared every two weeks and kept in a 4ºC fridge.

The coconut oil melts at room temperature which increases the risk of flies getting stuck in the food and dying. All vials were therefore kept in an 18ºC incubator. In addition to having strictly controlled temperature conditions, we attempted other measures to minimise the risk of mechanical death. All vials were kept sideways to reduce the amount of time the flies spend directly on the food. The high-fat food prepared out of the coconut oil is also more prone to dehydration which leads to the formation of cracks between the food and the walls of the vial. This often causes the flies to get stuck. Therefore, the high-fat vials were changed every three days to make sure the flies consume the fresh high-fat diet. Normal food vials weren't changed as often as the high-fat vials. They were changed only when the food started to show signs of dehydration which would be between 7 – 14 days.

Experimental Protocols

(i) Brain Homogenate preparations and quantification of the total head protein content

The preparation for the 96-well plate experiments involved the head removal and homogenisation procedure. Heads and supernatant were stored in a –80ºC refrigerator. 8 heads of each genetic line and condition (Fig. 11) were used in each 96-well plate experiment. Heads were homogenised in NaCl (150 mM), Tris of 8.0 pH (50mM), and EGTA (2mM). The homogenates were centrifuged at 5000 rpm for 5 minutes and the supernatant was collected for the assays. The total brain protein content was determined using a Thermo Scientific™ Pierce™ 660nm Protein Assay and the results of the biochemical assays were standardised against the total protein content. Greiner 96-well plates, black with clear bottoms, were used.

(ii) Quantification of total brain cholesterol concentrations

We used the Amplex Red Cholesterol Assay Kit (Invitrogen; A12216) to detect free cholesterol and cholesteryl esters. Cholesterol is produced when cholesteryl esters

are hydrolysed by cholesterol esterase. Cholesterol is then oxidised by cholesterol oxidase to release H_2O_2. In the presence of horseradish peroxide, the Amplex Red reacts with H_2O_2 to produce fluorescent resorufin. The samples were analysed in the PHERAstar Plate Reader using the FI 570 610 optic module.

(iii) Determination of lipid peroxidation

We followed the instructions of the Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, MAK085). The assay determines lipid peroxidation by the reaction of the thiobarbituric acid with malondialdehyde (MDA) which is an end-product of an oxidative attack on the polyunsaturated lipids. The fluorescent absorbance was measured in a Clariostar plate reader (excitation = 532 nm/emission = 553 nm)

(iv) Immunofluorescent labelling of Drosophila Mushroom Body neurons

We adapted an experimental procedure described by (Kelly, Elchert, & Kahl, 2017) to fluorescently label and image the mushroom body neurons. The final experimental protocol we produced had the following steps:

a) Anesthetize flies with CO_2 and remove fly heads using dissection scissors.
b) Dissect adult Drosophila brains in a PBS buffer solution (0.1M NaPhosphate,pH 7.2, 0.1% Triton-X-100).
c) Using p200 pipette, transferer one brain at a time to separate 0.5 mL microcentrifuge tubes filled with 30 uL of 4% paraformaldehyde diluted in PBS and incubated the brains for 20 minutes on a rocker.
d) Remove the fixative and performe three long washes 20 minutes each on a rocker in 30 uL of PBS.
e) Remove the last wash and incubate the brains at room temperature for 30 minutes in 30 uL of blocking solution [PBS + 5% NGS].
f) Remove the blocking solution and add 30 uL of the primary antibody (1D4 anti-Fas II-s, ordered from DSHB) diluted 1:20 in [PBS + 5% + 1D4] and incubate the brains for 1-2 nights at 4^{o}C.
g) Remove the primary antibody solution and perform three long washes of 20 minutes each in 30 uL of PBS.
h) Incubate the brains for three hours in Alexa Fluor 488 goat anti-mouse IgG (H+L) secondary antibody (Invitrogen a1101), diluted 1:200 in PBS + 5% NGS.

i) Remove the secondary antibody mixture and perform three long washes, 20 minutes each, in PBS.
j) Use a 20uL pipette to transfer each brain onto a microscope slide. Use a 10uL pipette to remove the excess buffer from the slide. If needed, use a piece of tissue to absorb any remaining fluid, leaving the brain and the slide as dry as possible.
k) Add a drop of fluorescent anti-fade mounting medium to each brain. Adjust the position of the brain using a stereomicroscope and a pair of forceps with antennal lobed facing up.
l) Place coverslips over the brains and seal it with the fingernail polish.
m) Visualised the Mushroom Body Neurons using Zeiss LSM880 confocal laser scanning microscope and computer software: ZEN Zeiss and ImageJ.

The confocal imaging of the brains would take place within 1-7 days. No coverslips were imaged after 7 days have passed to make sure we image only the freshest samples.

(v) Assessment of the blood-brain barrier integrity

We adapted a protocol described by (Love & Dauwalder, 2018) which is based on microinjection of the small-molecular-weight molecules into the open circulation of Drosophila and subsequent imaging of their permeation into the brain. The procedure involves the fluorescent labelling of the SPG cells using 10 kDa Dextran conjugated Texas-Red (TR) fluorophore (Invitrogen D-1863). The presence of the open circulatory system would allow the dye to circulate the body and reach its target BBB cells around the brain. Under physiological conditions, the BBB is intact which would make the injected dye accumulate around the brain. In contrast, the leaky BBB would allow the dye to diffuse into the brain. Following injection, the flies recovered overnight in the normal food vials and had their brains dissected ~12 hours later.

We have adjusted this protocol to complete the injection without the use of the microinjector. Instead, we employed a GENERAL VALVE PICOSPRITZER II MICROCELLULAR INJECTION UNIT which is capable of rapid and reproducible ejections of nanolitre volumes using air pressure. The following settings were used:

pressure of 60 psi and duration of 1 millisecond. The setup was connected to the pCLAMP Software which was used to trigger the stimulus.

Next, we used a Sutter micropipette puller (P-97) to manufacture sharp electrodes. The dextran dye has a higher density than water. Therefore, the needles have to be sharp enough to perform the injection with minimal physical damage, but wide enough to allow the dye to exit. The settings closest to the optimal size were as follows: Heat – 285; Pull – 20; Velocity – 70; Time – 150. The end of the needle tip was then broken using dissection forceps under the stereomicroscope. If broken correctly, the needle would be slightly wider, to allow the exclusion of the dye, but still suitable for injections.

The main objective was to test the volumes we inject by adjusting the settings on the Picospritzer. At present, we cannot confidently identify the exact volume we injected using the settings mentioned above. We modified the settings until we produced the close-to-optimal volume which would meet the main two requirements: the dye would successfully circulate through the body and the injection would not cause death.

The flies were anaesthetized on the frozen Petri dishes and positioned under the stereomicroscope. The manufactured needles could not penetrate the abdomen of the flies without causing too much physical damage, so we injected 2.5 mM Texas-Red Dextran, fixable in H_2O, into the chest. The dissection and immunohistochemistry procedure we followed was very similar to the one described in the protocol of the mushroom body staining. The brains were mounted onto the slides with an antifade DAPI mounting media for staining the nuclei. The slides were imaged using a Scientifica Slicescope microscope which was connected to the pE-4000 Illumination System for fluorescent microscopy (CoolLED). Texas Red Dextran was visualised using 633 nm wavelength and DAPI-stained nuclei at 405 nm.

(vi) Longevity

The experimental flies used for longevity were set up and kept separately to the flies used for other experiments. The 24-hour hatching window was recorded as the beginning of the longevity assay. A regular fly count continued until all the flies in the vials have died. Flies were disqualified and removed from the final sample count if

their death was caused by mechanical damage associated with coconut oil or vial transfer. The longevity assay was performed on all genetic lines under normal and high-fat diet conditions. The w^{1118} flies on normal food are absent from the final count due to the accidental death of the line during the experiment. w^{1118} flies on high-fat diet are therefore used as a control group in this experiment.

Data Analysis

Confocal Image

Confocal images were taken using Zeiss LSM 880 confocal microscope. Z-stacks were produced to generate maximal projection images and saved as the czi files. The confocal Images were analysed, and the surface areas measured using ImageJ software.

Statistics

Statistical analysis was performed with Prism 9.4 (Graphpad Software Inc., San Diego, CA, USA). Two-way ANOVA test was used when applicable with a posteriori test (with Tukey's multiple comparisons). Cumulative survival curves (longevity) are presented and compared using the Log-rank (Mantel-Cox) test. Data are expressed as mean ± SEM where *n* is the number flies. Significance is shown as $*p < 0.05$, $**p < 0.01$, $***p < 0.01$ and $****p < 0.0001$, n.s. (not significant).

Results

Quantification of the Total Cholesterol Content in the Brain

For cholesterol quantification, we used a biochemical assay which is based on an enzyme-coupled reaction that detects free cholesterol and cholesteryl esters which constitute a considerable portion of cholesterol in the blood. We compared the cholesterol levels in each genetic line following exposure to the normal (NF) vs high-fat (HF) diet (Fig. 11A). We also report how cholesterol levels change on each diet by comparing cholesterol expression at different ages (Fig. 11B).

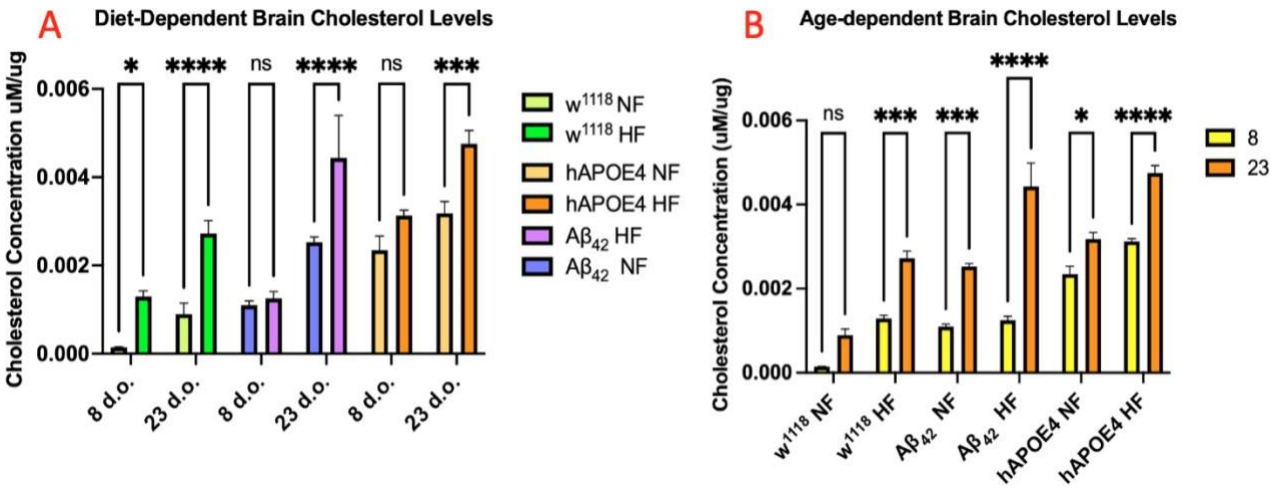


Figure 11: The effect of the age and HF diet on the total cholesterol brain levels in Drosophila flies with different genetic predispositions. (A) *Diet-induced differences in cholesterol concentrations. Showing a significantly higher cholesterol content on the HF diet across different mutations and ages, except for young hAPOE4 and Aβ42 flies.* ***(B)*** *Age-dependent differences in cholesterol concentrations. Showing that cholesterol levels increase with age for controls on HF and for AD mutants on both diets. Two=way ANOVA, data represent mean ± SEM, n= 8 heads.*

Control flies demonstrated a non-statistically significant increase in cholesterol content on NF (Fig. 11B), which suggests that cholesterol levels are maintained at constant levels under normal physiological conditions. In contrast, w^{1118} HF flies expressed higher cholesterol concentration at 8 days in comparison to the age-matched controls on NF (Fig. 11A). This data indicates that an increase in cholesterol levels can appear very shortly after the initial exposure to HF. The cholesterol levels kept increasing for w^{1118} as they aged on HF, reaching concentrations that were far above healthy levels (Fig. 11B).

hAPOE4-mutated Drosophila reflected the sporadic, Alzheimer's phenotype in humans. hAPOE4 NF flies had the highest cholesterol content at a young age compared to the other genotypes (*hAPOE4 vs w1118, ****p; hAPOE4 vs Aβ42, **p*). This finding suggests that even under normal dietary conditions and at a young age, hAPOE4 sporadic mutation increases susceptibility to high cholesterol content. This is not surprising, considering the role of the APOE4 gene in cholesterol trafficking. At 8 days old, there is no significant difference between hAPOE4 flies on NF and HF (Fig. 11A), suggesting that HF is not responsible for high cholesterol levels of hAPOE4 at a young age. However, as HF-fed hAPOE4 flies reach middle age, their cholesterol

pool is much above the age-matched hAPOE4 flies on NF (Fig. 11B). The results suggest that HF is responsible for high cholesterol levels of hAPOE4 in middle age, but not young age.

There is a certain degree of comparability between $A\beta_{42}$ familial mutants and hAPOE4. Firstly, $A\beta_{42}$ also express cholesterol levels on NF above the similarly young controls (**p)*. Secondly, the ageing of $A\beta_{42}$ on NF also demonstrates an increasing brain cholesterol pool (Fig. 11B). Abnormal expression of cholesterol in young brains suggests that the $A\beta_{42}$ have a predisposition towards cholesterol imbalance, similar to flies with the sporadic mutation. However, this predisposition appears to be weaker in $A\beta_{42}$ than in hAPOE4, once again emphasising the role of the APOE4 gene in cholesterol trafficking. Furthermore, we report that middle-aged $A\beta_{42}$ HF flies had severely increased cholesterol content following HF exposure (Fig. 11B), which matched the levels of hAPOE4. HF appears to have a similar effect on middle-aged familial and sporadic Alzheimer's mutants.

Overall, we report that HF has a significant effect on the cholesterol content in Drosophila brains. Diet-induced increase in cholesterol content follows relatively the same patterns for both Alzheimer's mutations. Interestingly, $A\beta_{42}$ and hAPOE4 did not show any significant difference between HF and NF at 8 days, unlike the control flies This suggests that there might be a mechanism which delays the effect of HF on cholesterol concentration in young Alzheimer's mutants.

Lipid Peroxidation

Our findings on lipid peroxidation levels complement our cholesterol data and illustrate how oxidative stress levels change following exposure to the HF diet (Fig. 12A) as well as age (Fig.12B). We used malondialdehyde (MDA) levels in Drosophila heads as a measure lipid peroxidation. MDA is one of the end products of the well-defined chain reaction which results from an oxidative attack on the polyunsaturated lipids.

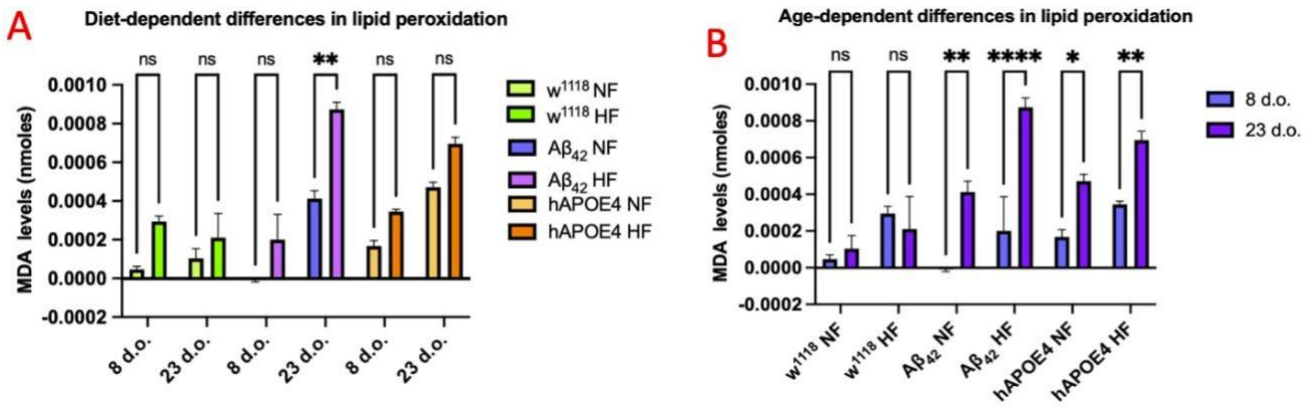


Figure 12: The effect of the age and the HF diet on the lipid peroxidation levels in Drosophila flies with different genetic predispositions. (A) *Diet-induced differences in MDA levels. No significant effect on MDA levels caused by the HF diet, expect for middle-aged Aβ42 flies.* ***(B)*** *Age-dependent differences in MDA levels. Showing that MDA levels increase with age for AD mutants on both diets, but not for controls. Two=way ANOVA, data represent mean ± SEM, n= 8 heads.*

As expected, NF w^{1118} flies expressed small levels of MDA throughout their life span which indicates the absence of oxidative stress-induced lipid degradation. Our data demonstrate that age does not induce lipid peroxidation in the healthy controls (Fig. 12B). Similarly, HF did not cause MDA release in controls at 8 or 23 days (Fig. 12A). Taken together, these findings indicate that healthy wild types are not vulnerable to an oxidative attack on the lipids caused by the poor diet or ageing.

NF $Aβ_{42}$ mutants have expressed an increased lipid peroxidation as they aged, with the MDA levels being significantly higher at 23 than at 8 days (Fig. 12B). However, the elevated MDA levels in neither young nor middle-aged NF $Aβ_{42}$ are above the healthy levels displayed by w^{1118} flies. Similarly, the MDA levels remained within the healthy range following 5 days of HF administration (Fig. 12A). On the other hand, long-term exposure to HF increased MDA production at 23 days compared to age-matched flies on NF (Fig. 12A) and even larger impact was observed when comparing 23 and 5 days on HF (Fig. 12B). The MDA levels of AB_{42} flies that followed HF diet most of theiradult life were over the healthy levels observed in w^{1118} flies (****p*).

Similar to the flies with the familiar mutation, hAPOE4 mutants displayed an increased MDA production at 23 days compared to 8 days old under normal dietary conditions (Fig. 12B). However, the MDA levels of young NF hAPOE4 did not differ from healthy

w^{1118}. Furthermore, NF hAPOE4 displayed similar MDA levels to the $A\beta_{42}$ flies at both ages, suggesting that there are no severe oxidative stress events in AD mutants on normal food. In contrast, the long-term HF diet exposure does cause increased lipid peroxidation as the MDA levels were significantly elevated at 23 days, but not at 8 days (Fig. 12B). More importantly, this expression exceeds the levels of the age-matched wild-types (***p*) suggesting that following HF for a long time does bring the lipid peroxidation level above the healthy levels in the sporadic AD mutants. On the other hand, we report no difference between HF-induced NF-induced MDA levels in hAPOE4 (Fig. 12A) with MDA levels on HF matching the levels age-matched flies on NF. This may indicate that it is the ageing that is responsible for lipid peroxidation levels in the sporadic AD mutans, and not the diet.

Overall, our results indicate that sporadic and familial mutants share similarities in the levels of oxidative stress under normal dietary conditions. Consistent with their healthy phenotype, w^{1118} flies do not present with the signs of lipid degradation and HF does not initiate this process. In contrast, long-term exposure to HF aggravates the lipid peroxidation levels in hAPOE4 and $A\beta_{42}$ which would otherwise remain within a healthy range. However, only $A\beta_{42}$ mutants presented with the HF-dependent MDA release, suggesting that the HF diet is only responsible for lipid peroxidation in the familial mutants.

The increased MDA levels of the sporadic mutants, on the other hand, were more likely to be caused by ageing rather than HF. Due to the later onset phenotype, hAPOE4 may have a better defence mechanism which protects the flies from diet- induced oxidative stress in middle age. In contrast, $A\beta_{42}$ flies present with an early disease onset and their defence mechanisms may already be impaired in middle agemaking them more susceptible to HF-induced effects.

Immunofluorescent Staining of Mushroom Body Neurons

We have imaged Mushroom Body (MB) neurons which have central location in the fly brains and are essential for learning and memory. These neurons are regarded as the major processing centre, capable of mediating the encoding, consolidation, and retrieval of odour information (Busto, Cervantes-Sandoval, & Davis, 2010). MB structure consists of the three lobes also known as the α-, β-, and γ -subunits. We visualised MD structured using an Fasciculin 2 (Fas2) antibody. Fas 2 is a cell-cell

adhesion protein which is expressedin high levels in the MB lobes. We analysed the morphological appearance of the lobesand the average surface areas of the subunits, comparing them following NF vs HF diet (Fig. 13 A,C,E) and at two different ages (Fig. 13 B,D,F).

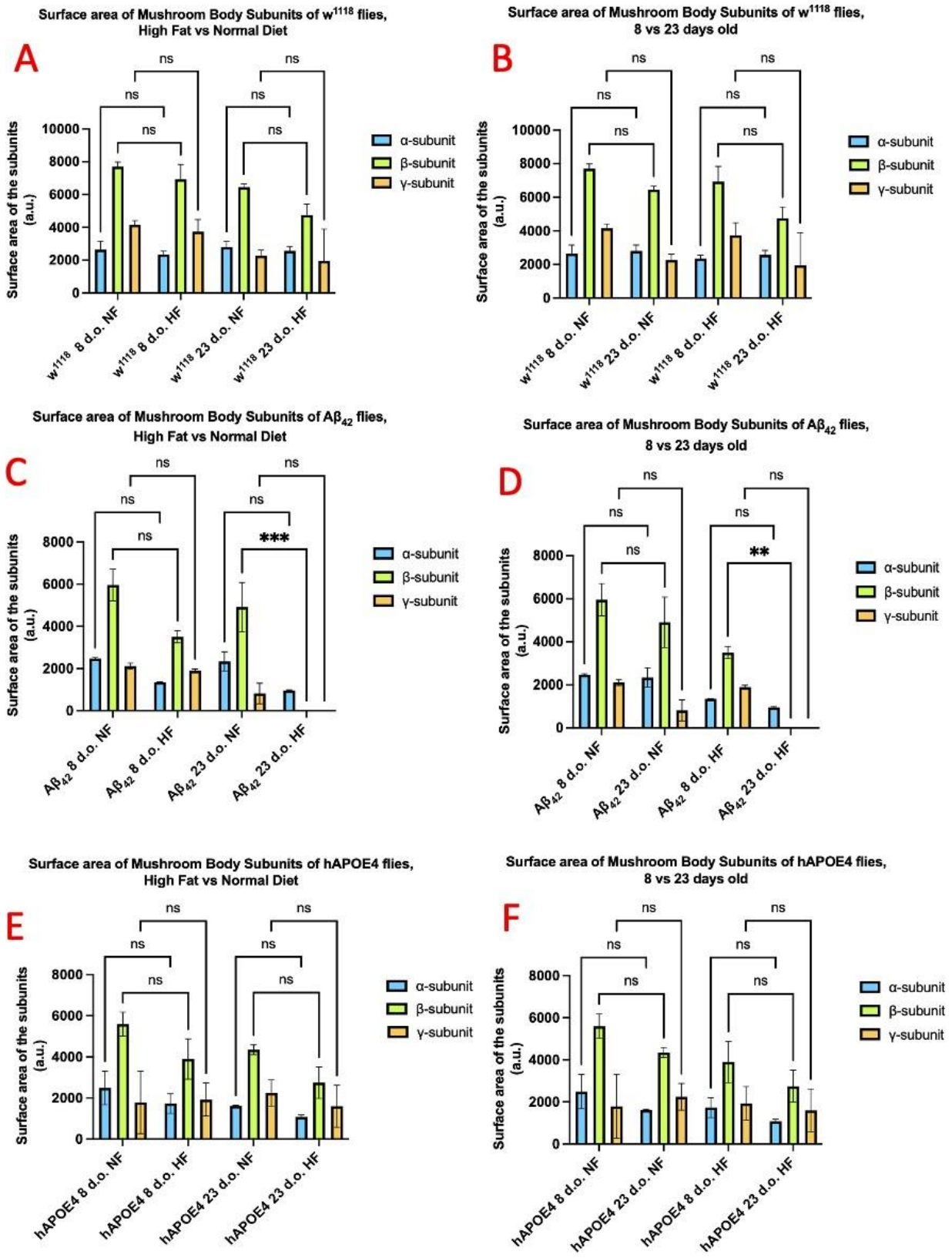


Figure 13: Comparison of the MB subunits of w1118, Aβ42, and hAPOE4 flies. *(A),(C),(E), MB following NF vs HF diet; (B),(D),(F) MB subunits at 8 vs 23 days of age. Two=way ANOVA, data represent mean ± SEM, n= 3.*

w1118 Mushroom Body Morphology

w1118 NF showed no signs of age-induced neurodegeneration (Fig. 14A,C) which is consistent which their healthy phenotype. The images also revealed no structural differences following either short-term or long-term exposure to the HF diet (Fig. 14B,D) which would be indicated by the reduced surface area of MB in maximal projection images. According to the statistical analysis of the surface areas, not a single MB subunit of the w1118 flies showed a sign of deterioration as we report no significant difference between the subunits following their transition into middle age (Fig. 13B). Similarly, the surface area deterioration was not in any way facilitated by the administration of the HF diet (Fig. 13A).

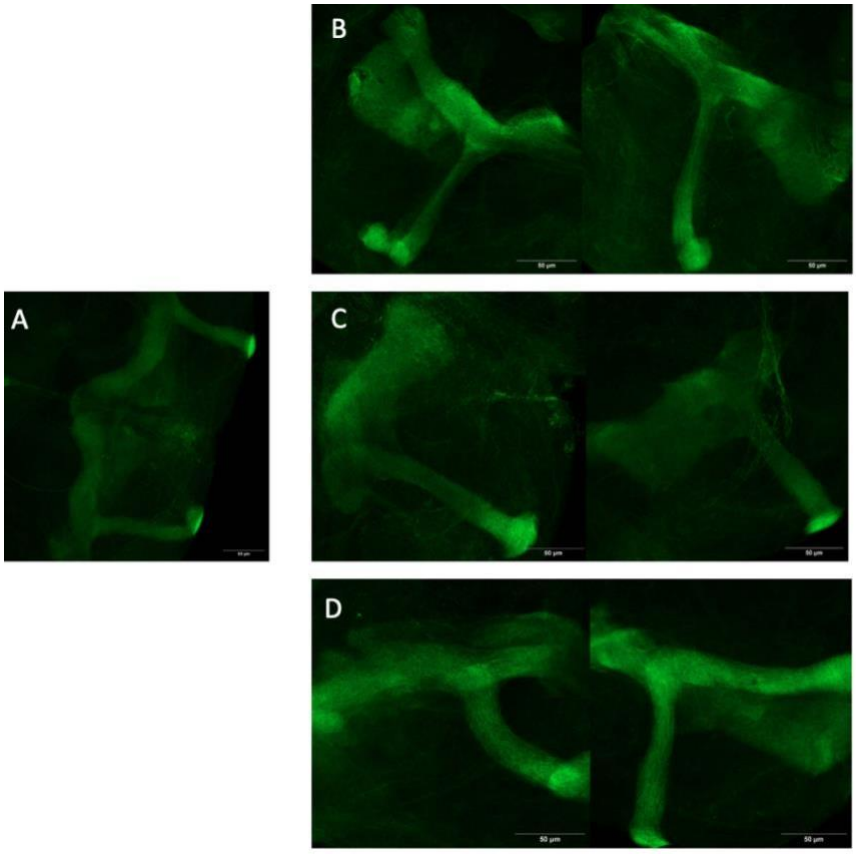


***Figure 14: Confocal images of the w1118 MB neurons and the average surface areas of subunits (a.u.)*. (A)** *W1118 NF (8 d.o.) α-subunit = 2644, β-subunit = 7695, γ-subunit = 4151;* **(B)** *W1118 HF (8 d.o.) α-subunit = 2352, β-subunit = 6929, γ-subunit = 3740;* **(C)** *W1118 NF (23 d.o.) α-subunit = 2808.26, β-subunit = 6452, γ-subunit = 2274;* **(D)** *W1118 HF (23 d.o.) α-subunit = 2568, β-subunit = 4750, γ-subunit = 1946.*

Aβ$_{42}$ Mushroom Body Morphology

Young NF Aβ$_{42}$ mutants displayed a similar MB structure to w^{1118} flies which rules out the presence of neurodegeneration at the early age (Fig. 14A, Fig. 15A). However, we begin to detect structural deformation of the α-subunit at the age of 23 days on NF (Fig. 15C), but these changes in the visual appearance are not accompanied by a significant reduction of the surface area (Fig. 13D). Interestingly, we can observe significant structural deformations in all the three subunits as early as at 8 days on the HF condition (Fig. 15B). However, the visual malformation of the subunits once again does not co-occur with the surface area reduction (Fig. 13E). The only significant decrease in the surface area is reported following long-term HF exposure in the β-subunit, which is the largest component of the MB. We report severe HF-induced degeneration at 23 days, compared to the age-matched flies on NF (Fig. 13C). Similarly, there is a significant difference in the extent of damage caused by short- and long-term exposure to HF (Fig. 13D), which once again is visible in the β-subunit. These changes are even more apparent on the confocal images of the MB where we can observe a significant loss of signal, deformation, and reorganisation of the subunits (Fig. 15D).

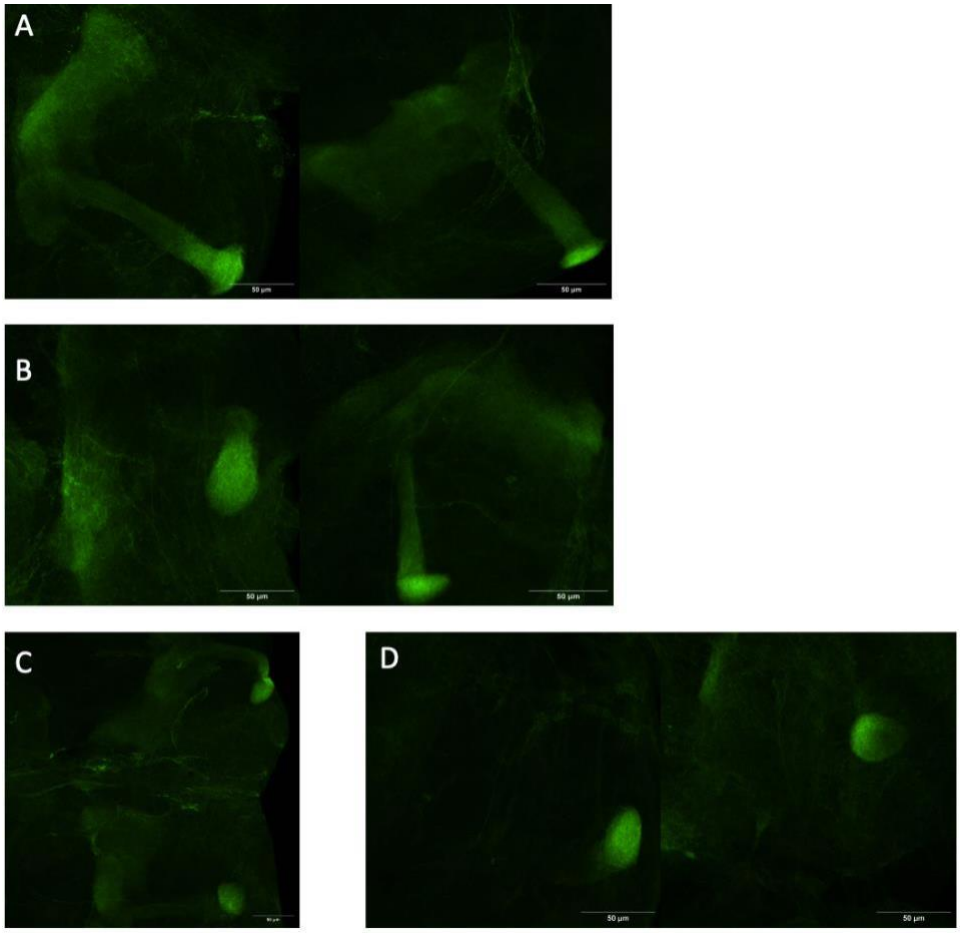


Figure 15: Confocal images of the Aβ$_{42}$ MB neurons and the average surface areas of the subunits (a.u.) (A) *Aβ$_{42}$ NF (8 d.o.) α-subunit = 2476, β-subunit = 5958, γ-subunit = 2113;* ***(B)*** *Aβ$_{42}$ HF (8 d.o.) α-subunit = 1358.469, β-subunit = 3505, γ-subunit = 1899;* ***(C)*** *Aβ$_{42}$ NF (23 d.o.) α-subunit = 2342, β-subunit = 4909, γ-subunit = 819;* ***(D)*** *Aβ$_{42}$ HF (23 d.o.) α-subunit = 959, β-subunit = 0, γ-subunit = 0.*

hAPOE4 Mushroom Body Morphology

Similar to w^{1118}, confocal images demonstrated no evident signs of neurodegeneration for hAPOE4 flies. There was no morphological change as the NF flies transitioned into middle age (Fig. 16A,C). The statistical comparison of the subunits revealed no significant alterations which would be caused by ageing (Fig. 13F). This is consistent with the late-onset phenotype of the hAPOE4 mutation when the symptoms manifest at an older age. We also report no structural alteration which would be caused by short-term exposure to HF (Fig. 16B). In turn, long-term exposure appears to have caused a slight deformation of the β- and γ-subunits (Fig. 16D) which could indicate that HF had sped up the early neurodegenerative processes which would otherwise

begin much later in time. However, the statistical comparison revealed no significant alterations caused by HF diet administration (Fig. 13E).

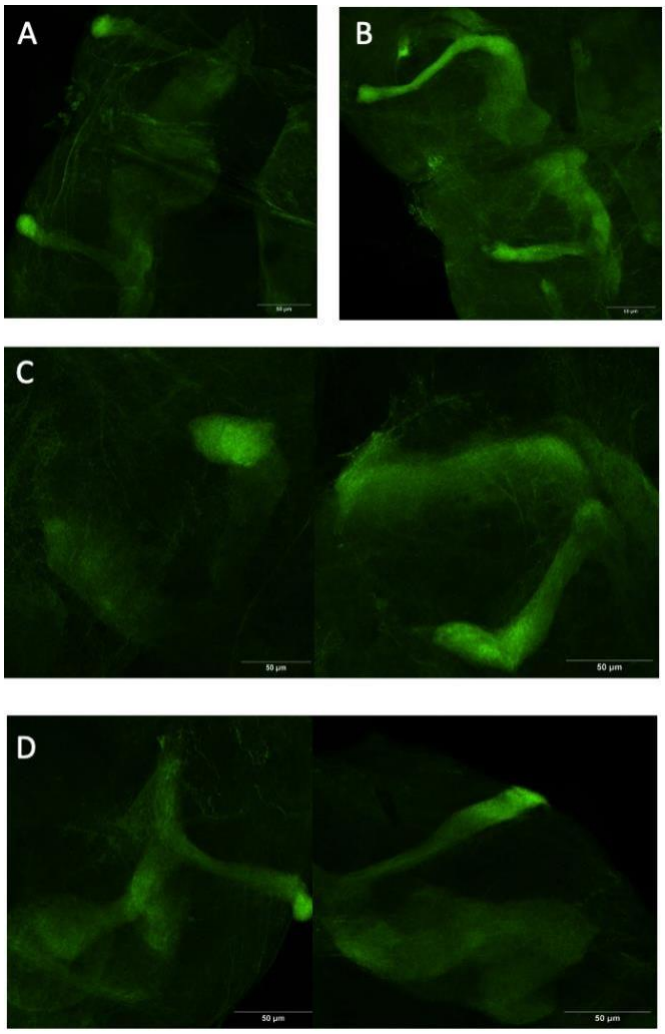
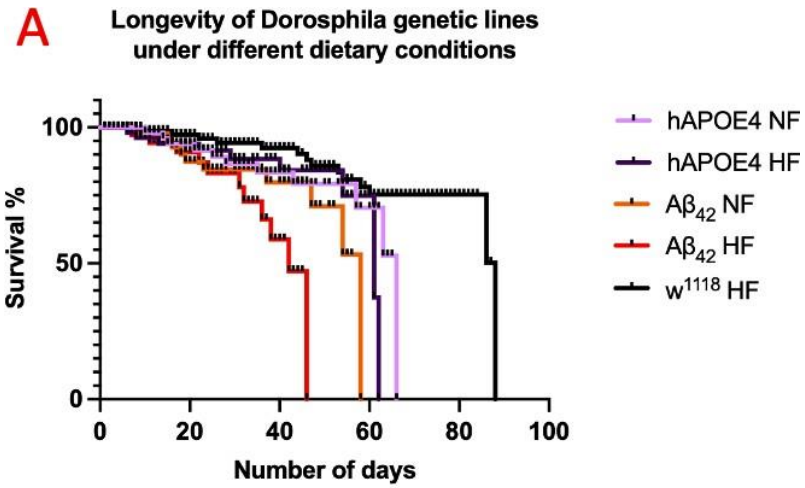


Figure 16: Confocal images of the hAPOE4 MB neurons and the average surface areas of the subunits (a.u.).
(A) *hAPOE4 NF (8 d.o.) α-subunit = 1611, β-subunit = 4347, γ-subunit = 2241;*
(B) *hAPOE4 HF (8 d.o.) α-subunit= 1728, β-subunit=3894, γ-subunit= 1925;*
(C) *hAPOE4 NF (23 d.o.) α-subunit=2491, β-subunit = 5595, γ-subunit = 1785.828;*
(D) *hAPOE4 HF (23 d.o.) α-subunit = 1082, β-subunit = 2745, γ-subunit = 1598.*

Longevity

We report the longevity trends of all the Alzheimer's lines under different dietary conditions (Fig. 17A). The Log-rank (Mantel-Cox) statistical test was used to calculate the statistical significance between the survival curves (Fig. 17B,C). The HF diet w^{1118} flies were used as a control group.

A



B

AD mutant groups on NF vs HF	P value	P value summary
$A\beta_{42}$ NF vs $A\beta_{42}$ HF	0.0499	*
hAPOE4 NF vs hAPOE4 HF	0.6338	ns

C

Other genetic groups	P value	P value summary
w^{1118} HF vs $A\beta_{42}$ HF	<0.0001	****
w^{1118} HF vs hAPOE4 HF	0.1113	ns
hAPOE4 HF vs $A\beta_{42}$ HF	0.007	**
hAPOE4 NF vs $A\beta_{42}$ NF	0.0146	*

Figure 17: Survival Trend of Drosophila on HF vs NF diet. ***(A)*** *Median Survival values: w^{1118} HF = 88 days; hAPOE4 NF = 66 days; hAPOE4 HF = 61 days; $A\beta_{42}$ NF = 58 days; $A\beta_{42}$ HF = 42 days.* ***(B)*** *Survival differences between NF and HF for $A\beta_{42}$ and hAPOE4.* ***(C)*** *Survival differences between different genetic groups on different diets.*

HF $A\beta_{42}$ presented with the lowest median survival age of 42 days, which is 16 days less that for the same genotype under normal food conditions (Fig. 17A). The statistical comparison between the conditions indicates that the HF condition significantly reduced the lifespan of $A\beta_{42}$ flies (Fig. 17B). Furthermore, the lifespan of $A\beta_{42}$ flies was significantly reduced than in w^{1118} controls on HF (Fig. 17C).

On the other hand, the survival of the hAPOE4 mutants does not appear to be affected by HF conditions. The difference in median survival of hAPOE4 flies on normal compared to HF diet is only 5 days (Fig. 17A). This is reflected in the non-significant difference between their survival curves (Fig. 17B). We also report no statistical difference between the survival curves of the hAPOE4 and w^{1118} controls (Fig. 17C).

Despite the lack of significance when compared to controls, hAPOE4 reported a significantly higher survival rate than $A\beta_{42}$ flies on NF and HF (Fig. 17C).

Assessment of the Blood-Brain Barrier Permeability

We have successfully stained the SPG cells which form an inner layer of Drosophila BBB. Figure 18 shows the Dextran-TR labelled SPG cells accumulating at the barrier. One of the brains (Fig. 18D) still has a portion of an eye attached. The Dextran-TR can be seen successfully surrounding the eye, separating it from the brain. We also demonstrate the DAPI-stained nuclei inside the brain in addition to the SPG cells and we can see a good DAPI staining of the nuclei inside the brain (Fig. 18C,D). However, we can also observe a scattered fluorescent signal, albeit a weaker one, coming from inside the brain on all the images. This signal could possibly be the Dextran-TR dye crossing the barrier into the brain, which would be expected to happen if the integrity of the BBB was compromised.

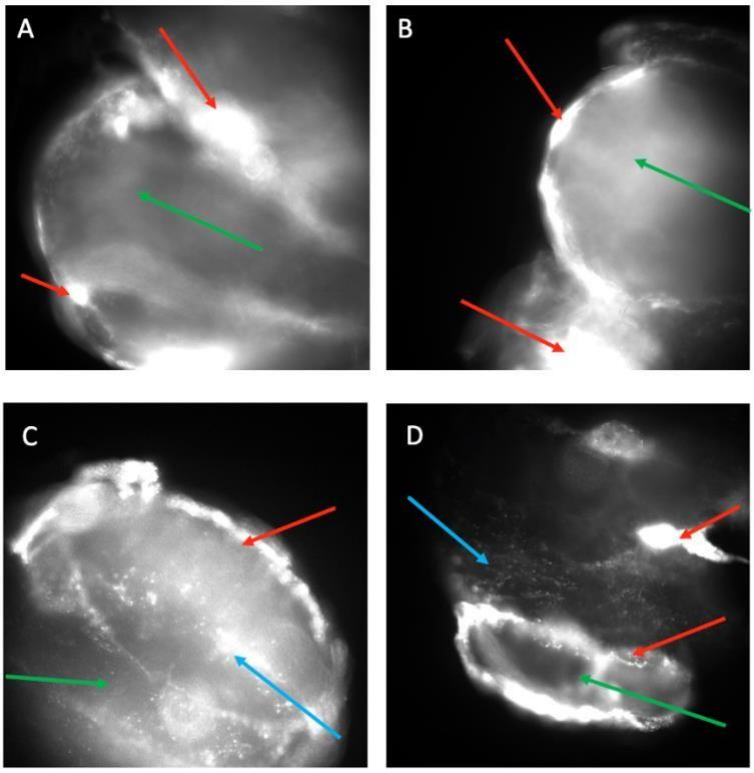


Figure 18: Dextran-TR labelled SPG cells of the BBB and DAPI-stained nuclei. *Dextran-TR was visualised at 633 nm and DAPI at 403 nm excitation. Red arrows point to the accumulation of Dextran-TR at the barrier. Blue arrows point at the neuronal nuclei. Green arrows point to the Dextran-TR signal within the brain possibly caused by diffusion through the BBB into the brain.*

Future directions – memory and learning testing

The next stage of the research we started to develop was the assessment of cognitive performance. We have adapted an existing protocol and designed a T-Maze which can be manufactured using a 3D Printer.

Memory and learning impairments are recognized as the main hallmarks of neurodegenerative progression. Drosophila has once again proved to be a convenient model for the assessment of cognitive behaviours such as learning and memory. The conserved mechanisms of olfactory learning between flies and mammals (Matsunami & Amrein, 2003; Y. Wang, Pu, & Shen, 2013) allow us to study the mechanisms of conditioning behaviours. In addition, the ease with which the olfactory system of Drosophila can be manipulated in a lab setting made it the most widely employed technique in studies that aim to assess the learning and memory of the flies. Therefore, multiple olfactory memory paradigms have been developed to study the memory encoding and retrieval neural mechanisms (Ali, Escala, Ruan, & Zhai, 2011; Pitman et al., 2009) and have been adapted for use in different research questions.

The first assays to be developed using the principles of olfactory conditioning were a negative reinforced operant conditioning paradigm (QHB) (Quinn, Harris, & Benzer, 1974) and a negative associative Pavlovian conditioning assay (TQ) by (Tully & Quinn, 1985). Thefirst assay paired one of the two odours to an electric shock, teaching the flies to avoidthe odour which was associated with an aversive stimulus. The second paradigm has been further developed to teach flies to negatively associate a conditioned stimulus (i.e. odour 1) and an unconditioned stimulus (i.e. electric shock). In contrast to the firstconditioned odour, the second odour is introduced without exposure to the aversive stimulus during a training session. During the testing, flies are allowed to choose a tube with one of the two odours and the number of flies is counted in each case. It hasbeen reported that following training, the memory based on the olfactory conditioningpersists for further 24 hours. After being described by these two assays, the principlehas been adapted and customized by numerous studies and remains one of the mostcommon techniques for the memory and learning assessment of Drosophila.

We attempted to develop a slightly different olfactory conditioning protocol. It has been suggested that an aversive stimulus may not be an ecologically relevant stimulus for Drosophila in their natural environment (Pitman et al., 2009). Therefore, we sought to avoid the formation of memories that result from olfactory avoidance and aversive conditioning. Instead, we wanted to facilitate the formation of associative memories with the use of a reward stimulus. We suggest an olfactory appetitive conditioning paradigm adapted from (Krashes & Waddell, 2008) which would test the Drosophila associative memory in a T-Maze manufactured with the use of a 3D printer.

When performing this memory testing protocol alongside other experiments described in this research, we suggest keeping separate Drosophila lines for all experiments. Memory assays usually require the flies to undergo a starvation period. Therefore, we do not recommend using these flies for the biochemical assays. Starvation period could interfere with the results from the biochemical assays due to the well-documented effect that starvation has on the lipids (Chauhan, Anis, & Chauhan, 2021) and oxidative stress levels (Belyi et al., 2020; Tettweiler, Miron, Jenkins, Sonenberg, & Lasko, 2005) in Drosophila.

We propose that the training session takes place outside the T-Maze, which should only be used for testing. For the training session, two sets of vials should be prepared. One set of vials should contain agar solution while the other contains agar solution mixed with sucrose. Carrying out the training and testing sessions in different environments could be a potential limitation as this introduces an environmental factor. Therefore, both procedures should be conducted in similar and highly controlled conditions (i.e. light, temperature, humidity). On the other hand, having different environments for training and testing could be potentially beneficial since conducting training sessions in vials allows an easier and more efficient way of transferring the flies between the training sessions. Secondly, we eliminate the possibility of flies showing a preference for one of the wings because they were earlier introduced a positive conditioned stimulus in them. However, further research is needed to see if there is a difference in the formation of associative memories depending on where the testing sessions take place.

We propose the use of the AM odour (n-amyl acetate) paired with the sucrose reward

as a positive conditioned stimulus (CS+) and simple agar as an unconditional stimulus (CS). Before the experiment, the flies should undergo a period of starvation for 16-20 hours.

The experiment should be conducted with two repeats with at least 15 flies being used in each trial. During the training session, the flies should be transferred into a vial with the CS+ for 2 minutes. The flies should then be transferred into the training vial with only agar present and no smell (CS-) where they should be kept for 1 minute. Such transfers should be repeated two more times with fresh vials being used for such transfer (6 vials in total: three vials with CS+ and three vials with CS-) (Figure 19)

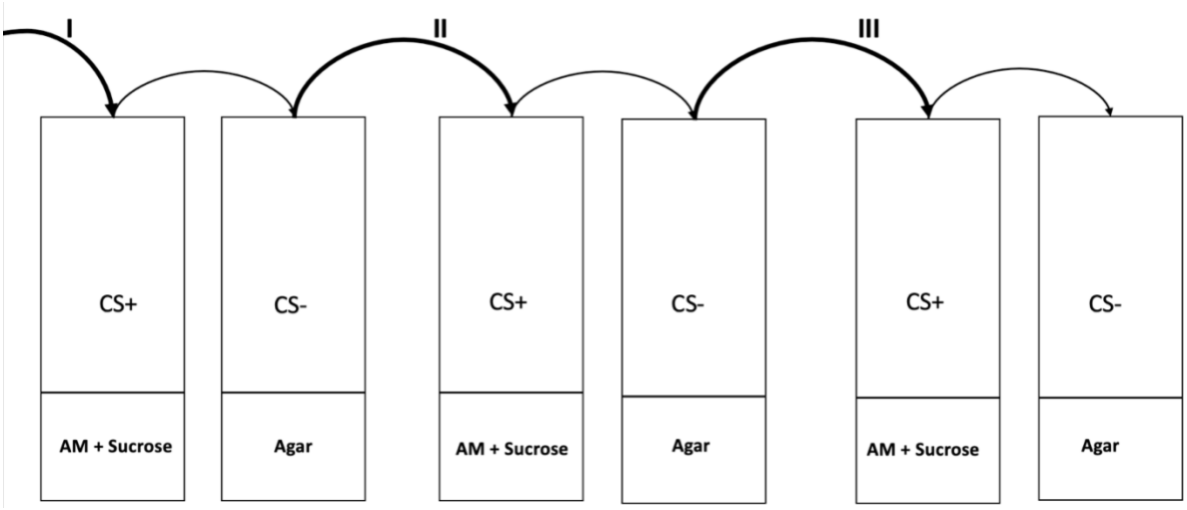


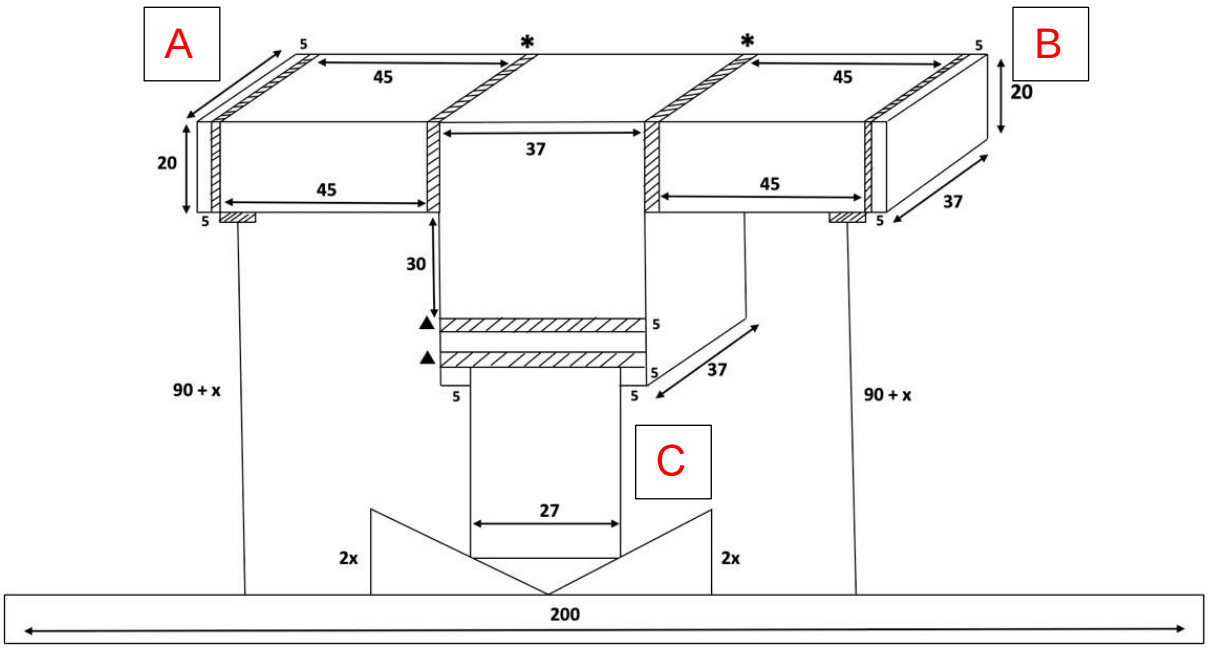
Figure 19: The training session, presenting AM odour paired with sucrose as a positive reward.

Alzheimer's disease is known to produce both short-term and long-term memory deficits, with the symptoms appearing in the early and late stages (Zverova, 2019). Therefore, to have a better understanding of cognitive performance, two-time points are equally important and required two testing sessions: the first testing session should take place immediately after the training session and the second testing session should follow ~17-20 hours later.

Figure 20 illustrates the design of the T-Maze prototype engineered for 3D printing. To begin the testing session, a filter paper should be placed on either one of the wings and a vial with the flies should be inserted into the T-Maze, as shown in the picture. The first sliding door should be opened, and a recovery time of one minute be given to the flies. The opening of the second door marks the start point of the experiment.

Before its opening, the T-Maze should be tapped slightly, to prevent injuring the flies during the door opening. The flies should be allowed 30 seconds to choose either wing, after which the door to the wing containing CS+ will be closed. Extra care should be taken not to injure the flies with the closing door. The flies that were lost due to mechanical injury should be disqualified from the experiment. CO_2 should then be released into the CS+ wing through the outside door, and flies collected and counted. The performance index is calculated as the number of flies choosing the conditioned wing minus the number of flies choosing the CS- wing, divided by the total number of flies used in the experiment excluding the flies that were lost during the experiment.

Before the training session takes place, it is important to understand the natural attraction that the flies have for the smell and investigate if there are any differences in their non-conditioned attraction to the odour. The innate attraction testing can also take place in the T-Maze following a similar protocol but without the sucrose reward. By presenting a smell in one of the wings, we can observe the number of flies that are attracted to the smell and move towards it. This data can then be used to calculate an AM preference using the following formula PrefAM = (#AM - #EW)/ #Total, where #AM is the number of flies that moved into the wing with the AM smell; #EW is the number of flies from the empty wing without the smell, and #Total is the total number of flies used in the test. A number between +1 and -1 is calculated which indicates the presence or the absence of innate attraction, respectively.



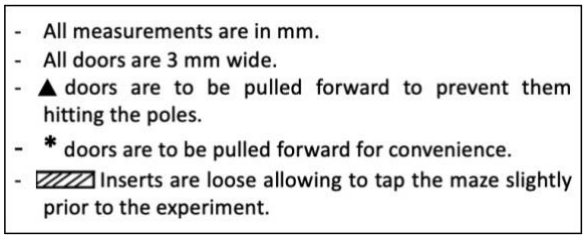


***Figure 20: Sketch of the T-Maze design**. **(A)** and **(B)**: the wings that contain either CS+ or CS-; **(C)**: a vial with the flies which should be inserted into the T-Maze.*

Discussion

We introduced a model that describes the mechanism through which the high-fat diet may affect the progression of Alzheimer's. This is a complex neurodegenerative disease with numerous predisposing factors including genetics, environment, and lifestyle. Following an increasing amount of research into the role that the Gut-Brain Axis plays in brain disorders, the high-fat diet has emerged as one of the major risk factors for dementia and neurodegenerative pathologies. Such dietary habits were found to influence multiple events which precede and accompany the disease, such as neuroinflammation, metabolic, and oxidative stress.

Cholesterol has been recognised as one of the mechanisms that allows diet to exert influence on these processes. Under normal physiological conditions, cholesterol carries out important functions in the brain while its biosynthesis and metabolism are strictly controlled by internal homeostasis mechanisms. The source of brain cholesterol is almost strictly endogenous with the astrocytes being the primary production site in adult brains. This is further facilitated by the BBB which successfully keeps the peripheral cholesterol out of the brain. An internal control system monitors the cholesterol balance either by inhibiting further biosynthesis or by initiating one of the four metabolism pathways: 1) esterification and subsequent intracellular storage in lipid droplets, 2) direct excretion via ABC transporters, 3) conversion into the 24-OHC or, 3) to a lesser extent, into the 27-OHC (Gamba et al., 2021). Under normal physiological conditions, the balance of oxysterols remains as follows: there is an exclusion of 24-OHC from the brain and an influx of 27-OHC into the brain. The disruption of this cholesterol homeostasis mechanism is linked to a higher risk of Alzheimer's disease onset and progression (Ito et al., 2019). There are also reports of increased levels of oxysterols contributing to the Aβ plaque aggregation through the

induction of neuroinflammation and oxidative stress (Ito et al., 2019). Furthermore, increased levels of cholesterol in the brain were directly linked to the switching of the nonamyloidogenic pathway into the Aβ-producing pathway through regulation of the APP proximity to β-secretase, γ-secretases (Kojro et al., 2001; H. Wang et al., 2021). One of the factors which is believed to be responsible for the accumulation of cholesterol and oxysterols in the brain is the reduced integrity of the BBB which we suggest could result from increased levels of oxidative stress.

We propose that the Western-like diet may be one of the major causes of increased cholesterol levels in the brain, thus contributing to the conditions that favour the amyloidogenic pathway. It was previously suggested that the fat-rich diet affects the signalling pathways involved in cholesterol biosynthesis and metabolism, thus disturbing internal homeostasis that regulates cholesterol levels. It is also possible, however, that high fat also contributes to the BBB damage by inducing gut dysbiosis and initiating inflammatory and oxidative stress cascade that ruins the structural integrity of the barrier. This would also compromise the barrier function and allow the entry of potentially harmful blood-derived compounds into the brain. Increased permeability could constitute another mechanism by which cholesterol originating elsewhere in the body diffuses into the brain and increases the cholesterol brain pool.

We aimed to develop an experimental approach which could be used to investigate the connection between the BBB, increased cholesterol brain content, neurodegeneration, and oxidative stress. We have attempted to adapt and optimise a series of experiments which would allow us to study these processes in young and middle-aged *Drosophila Melanogaster*.

Our results show that long-term exposure to the high-fat diet causes a dramatic increase in brain cholesterol levels across all Alzheimer's mutant lines. This increase is similar for both AD mutant groups, indicating that the diet affects both lines in a similar way. Most importantly, we reported no significant increase in cholesterol levels in our control group as they aged on normal food. This finding supports the notion that under normal physiological conditions, the brain maintains steady cholesterol levels through strict homeostasis mechanisms. Once we introduce long-term exposure to

unhealthy diet, the wild-type flies demonstrate an increase in brain cholesterol far above healthy levels. Our findings of cholesterol concentration in control wild-type flies are in line with previous experiments (Phillips, Woodruff, Liang, Patten, & Broadie, 2008). However, we found no traces of lipid peroxidation markers in w^{1118} following ageing and no evidence that the diet induces morphological neurodegeneration of the MB. On one hand, this is consistent with their healthy phenotype. On the other hand, this raises questions about the mechanism which would be responsible for the increased brain cholesterol content. Taking into accounttheir healthy phenotype and the absence of lipid degradation, we may expect their BBB to have healthy structural integrity. Furthermore, it is believed that Drosophila is incapable of *de novo* cholesterol synthesis in the brain (Tschape et al., 2002), which means that there cannot be any diet-induced upregulation of ongoing physiological cholesterol synthesis. Therefore, an alternative mechanism must exist in the wild-type Drosophila brain which would allow the entry of free cholesterol into the brain. Vertebrate BBB expresses several lipoprotein receptors which transcytose both low- density lipoprotein (LDL) and high-density lipoprotein (HDL) (Brankatschk & Eaton, 2010). Even though free cholesterol cannot cross the intact BBB by itself, cholesterolpresent on lipoproteins and oxysterols can be transported across the barrier (Rhea & Banks, 2021). Drosophila and vertebrates have highly similar lipoproteins and lipoprotein receptors as well as the BBB with an almost identical molecular structure (Brankatschk & Eaton, 2010; Mayer et al., 2009). There are currently no published studies which would investigate the mechanism of lipoprotein-mediated cholesterol transport in Drosophila. Further research into the effect of the high-fat diet on lipoprotein transport would be beneficial to account for the unwanted cholesterol entering the brains of flies with an intact BBB. In humans, one of the other mechanisms which could potentially be responsible for increased cholesterol brain content with anintact BBB is the upregulation of cholesterol *de novo* biosynthesis by the brain-derived neurotrophic factor (BDNF) (Petrov, Kasimov, & Zefirov, 2016; Spagnuolo et al., 2018). However, there is controversial evidence regarding the exact effect that the high-fat diet has on BDNF. Multiple studies report that a diet rich in high fat increases the expression of BDNF (Ramalho et al., 2018; Virtuoso et al., 2021). Other evidence points to the reduced levels of BDNF following high-fat diet exposure (Molteni, Barnard, Ying, Roberts, & Gómez-Pinilla, 2002; Molteni et al., 2004).

We also found no signs of neuronal degeneration in the wild-type flies and no signs that the high-fat diet contributed to any neurodegenerative-related events. Despite the evidence that hypercholesterolaemia can induce oxidative stress at the BBB (Banks & Rhea, 2021), we might not have observed any oxidative stress events because they did not yet present in middle-aged Drosophila. There are strong accounts of high cholesterol presence in mid-life being associated with a higher risk of AD much later in life (Dias, Polidori, & Griffiths, 2014). Therefore, investigating the morphology of the mushroom body and the lipid peroxidation levels at later stages would help confirm this. At present, our findings indicate that diet-induced cholesterol accumulation does not cause any severe neurodegenerative events in the middle-aged wild-types. On the other hand, we cannot completely rule out the possibility of oxidative stress in these flies. Even healthy ageing in wild-types is inevitably accompanied by oxidative stress (Fleming, Reveillaud, & Niedzwiecki, 1992). It would be useful to look at the effect that a high-fat diet has on oxidative stress in more detail, and study other markers of oxidative stress, such as Superoxide Dismutase and Reactive Oxygen Species expression.

The Aβ mutation represented the most severe cases of Alzheimer's – a highly penetrant familial mutation which manifests with an early onset of the disease. At a young age, this genotype was already expected to present with some predisposition to neurodegenerative events. Physiological levels of cholesterol in the brain of these young flies were significantly higher than in the healthy controls and the levels continued to grow as the flies aged. This could be an indicator of a genetic predisposition towards an imbalanced cholesterol content. Once again, the lipoprotein transport system in the Drosophila brain is likely to be involved, but there are currently no reports of Drosophila $A\beta_{42}$ mutants having dysfunctional lipid transport which would account for physiologically high cholesterol brain content. Long-term high-fat administration caused an even bigger jump in cholesterol levels which were significantly higher than the ones observed during simple $A\beta_{42}$ ageing. We can therefore conclude that high fat is capable of fuelling hypercholesterolaemia in familial mutants and potentially aggravating the toxicity associated with it.

What is more, the high-fat diet had a detrimental effect on all the other measures of neurodegeneration. Following high-fat exposure, increased cholesterol content in

middle age was accompanied by increased levels of lipid peroxidation and severe morphological degeneration of the biggest MB subunit. Ageing increases Drosophila's vulnerability to $A\beta_{42}$-induced toxicity (Rogers et al., 2012). But our data suggest that neurodegeneration in our middle-aged $A\beta_{42}$ flies was caused by high-fat administration and not ageing. These findings are consistent with our prediction that increased lipid peroxidation in familial mutants would be associated with greater cholesterol expression in the brain and worse neurodegeneration. Finally, despite an already shortened lifespan of $A\beta_{42}$ mutants, the high-fat diet decreased life expectancy even further. Previous evidence suggests that high fat has a negative effect on the Drosophila life span (Liao, Amcoff, & Nassel, 2021). Our research provides evidence that this impact is even stronger on the flies with the predisposing familial mutation.

hAPOE4 genotype represents LOAD cases. Due to its function as a coding gene of ApoE4 transport protein, APOE4 expression is strongly associated with the altered circulating cholesterol mechanisms. Mutations in this gene are linked to impaired cholesterol biosynthesis and abnormal accumulation in the lysosomes (Tcw et al., 2022), metabolism (Mann et al., 2004), and trafficking (Jeong, Lee, Cho, & Seo, 2019). The overallquantity of the lipid droplets is also higher in the ApoE4-rich astrocytes (Farmer, Kluemper, & Johnson, 2019). Not surprisingly, we observed the highest levels of physiological cholesterol in the young flies with this mutation. The high-fat diet increases the cholesterol levels inthe flies with the LOAD mutation even more. It is currently unclear whether high-fat diets can directly affect *de novo* cholesterol synthesis, but there are multiple accounts of high-fat increasing oxidative stress at the BBB and damaging its integrity (Onyango, 2021; Song & Li, 2020). We showed an increase in lipid peroxidation levels following long-term exposure to high fat in hAPOE4 flies, but these levels were similar to the levels observed under normal dietary conditions. Therefore, the increased lipid peroxidation in hAPOE4 flies is likely to be caused by ageing rather than high-fat. ApoE4 isoform is linked to increased lipid peroxidation rates, although the exact mechanism of this is unclear (Belaidi et al., 2022; Ramsden et al., 2022; Thorwald, 2022). On the other hand, we might not be able to rule out the possibility of high fat increasing lipid peroxidation levels in these mutants. The fact that we observed an increase in lipid peroxidation and MD neurodegeneration in EOAD flies, but not LOAD, is consistent with the Alzheimer's progression timeline. However, MBs displayed slight deformations of two of the

subunits following high-fat exposure, which could signify that the diet sped up the neurodegeneration in hAPOE4 flies. Despite this, high fat appeared to have no significant impact on the longevity of LOAD flies. These findingsare supported by previous studies indicating a much later onset of the disease in hAPOE4 flies (Haddadi, Nongthomba, Jahromi, & Ramesh, 2016) and greater longevity (Iijima et al., 2008) in contrast to EOAD which can cause neurodegenerative events as early as the beginning of middle age (Ling, Song, Garza, Neufeld, & Salvaterra, 2009).

Overall, the adaptation of the BBB protocol has worked well. We have successfully replaced the microinjector with the Picospritzer which could deliver the dye into the system. The protocol for the needle manufacture using a micropipette puller is also in working condition as it allows injection of the dense fluid without causing severe physical damage. We have also managed to image the slides using an electrophysiology set-up instead. Despite the images being black and white, the structures can easily be distinguished, and diffusion of the dye can also be observed. This protocol, however, requires further adjusting in terms of the setting of the pulse duration. We observed a weak signal coming from inside the brain which would potentially indicate the diffusion of the dye across the barrier. The experimental flies are expected to have an intact and fully functional BBB system. Therefore, one possible explanation is that the injected volume exceeded the recommended dose and overburdened the BBB. This would explain the unexpected entry of the dye as the high volumes could have overwhelmed the barrier causing the diffusion. The protocol would thus benefit from further optimisation to allow a more controlled way of the dye injection.

We also proposed a new memory and learning protocol which will allow us to study the long- and short-term memory deficits associated with different stages of Alzheimer's progression. It is widely reported that high fat negatively impacts memory and learning in a range of different disease models. However, there is a lack of evidence about the difference between short-term and long-term memory deficits. The protocol is based on the Drosophila olfactory nervous system which mediates olfactory memory and learning in Drosophila (Guven-Ozkan & Davis, 2014). Results from this experiment would complement the confocal analysis of mushroom body neurons due to their role in olfactory system. Specifically, the role of the mushroom body neurons

is to receive the information transmitted by the projection neurons and the local interneurons in the antennal lobe where olfactory information is signalled from the olfactory receptor neurons (Mariano, Achsel, Bagni, & Kanellopoulos, 2020; Yagi, Mabuchi, Mizunami, & Tanaka, 2016). They have been found essential for olfactory conditioning in flies irrespective of age and sex (Heisenberg, Borst, Wagner, & Byers, 1985).

The experiments have been successful at elucidating the effects of the high-fat diet on the markers of Alzheimer's neurodegeneration. Our findings have been consistent with the previous studies on the distinct pathological profiles in Alzheimer's mutants which points to the distinct molecular pathways involved in the progression of the disease. Only familial mutants reported high fat-dependent lipid peroxidation and reduced longevity. On the other hand, diet-induced increase in cholesterol content follows relatively the same patterns for both Alzheimer's mutations. Sporadic mutants also presented with lower degree of diet-induced neurodegeneration and no effect on longevity. We conclude that the high fat diet disturbs the brain cholesterol pool in both groups, but the effect on overall neurodegeneration in middle age is only evident in mutants with the familial genetic predisposition, due to much earlier disease onset.

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