IN VITRO AND *IN VIVO* STUDY ON THE IDENTIFICATION OF COMPOUNDS THAT ALLEVIATE Aβ42 ASSOCIATED NEURODEGENERATION

FLORENCE TAN HUI PING

UNIVERSITI SAINS MALAYSIA

2020

IN VITRO AND *IN VIVO* STUDY ON THE IDENTIFICATION OF COMPOUNDS THAT ALLEVIATE Aβ42 ASSOCIATED NEURODEGENERATION

by

FLORENCE TAN HUI PING

Thesis submitted in fulfilment of the requirements
for the degree of
Doctor of Philosophy

December 2020

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincerest gratitude towards Dr Ghows Azzam for the continuous support, advice and knowledge throughout my doctoral research. Also, to my co-supervisors, Prof Nazalan Najimudin and Prof Watanabe Nobumoto, thank you for the inspiration and insightful comments that have helped build my thesis.

Not to be forgotten, I extend my deepest appreciation to my fellow colleagues of Ghows Lab and Nazalan Lab. From long hours of executing experiments together in the laboratory to brain-storming on ideas, thank you for sharing your experiences with me. To Woo Wai Kan, Andrew Ting Chung Jie and the rest of the gang, it has been a joy working with every one of you.

Special thanks to the staff of USM's School of Biological Sciences and the Chemical Biology Research Group in RIKEN for their indispensable assistance in the project. I would also like to thank my esteemed sponsors MybrainSc and USM-RIKEN International Centre for Ageing Science (URICAS), for it is through their funding that has granted me the opportunity to further pursue my studies, both locally and internationally.

To my dearest parents and family who have been with me from the very start, there are no words to express my gratitude for the love, patience and sacrifices made to get me where I am now. Last but not least, thank you to my partner and lifelong friend, Tan Siew Heng for the encouragement and affection that you have showered me throughout my journey.

I would not have made it this far without any of you. Thank you for everything.

TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

µ	micro
ACE	Angiotensin converting enzyme
AD	Alzheimer's disease
ADI	Alzheimer's disease International
AICD	APP intracellular domain
AMP	Antimicrobial peptides
APOE	Apolipoprotein E
APP	Amyloid precursor protein
ATP	Adenosine tri-phosphate
Aβ	Amyloid-beta
BACE	Beta-secretase
Bgm	*Bubblegum*
BP	Biological processes
CAFÉ	CApillary FEeder
CC	Cellular components
CNS	Central nervous system
Co2+-CMA	Co(II)-carboxymethylaspartate
CYP	Cytochrome P450
dAPPl	*Drosophila* APP-like
dBACE	Beta-secretase-like enzyme
DEG	Differentially expressed gene
DMEM	Dulbecco`s Modified Eagle Media
DMSO	Dimethyl sulfoxide

dPS	*Drosophila* presenilin homolog
drd	*Drop-dead*
dTau	*Drosophila* tau
DWE	Danshen water extract
EGFR	Epidermal growth factor receptor
elav	Embryonal lethal, abnormal vision
EOFAD	Early onset familial Alzheimer's disease
ERK	Extracellular-signal-regulated kinase
FDA	United States Food and Drug Administration
FDR	False discovery rate
GMR	Glass Multiple Reporter
GO	Gene Ontology
GST-tagged	Glutathione S-transferase-tagged
H_2O_2	Hydrogen peroxide
HST	Heat shock protein
Ibs	Inclusion bodies
iCa2+	Intracellular Ca2+
IL	Interleukin
IMAC	Immobilized Affinity Column Chromatography
IPTG	Isopropyl β-D-1-thiogalactopyranoside
KEGG	Kyoto Encyclopedia of Genes and Genomes
LB	Lysogeny broth
LOAD	Late onset Alzheimer's disease
log2FC	log2FoldChnage
m	mili

MAPK	Mitogen-activated protein kinase
MF	Molecular functions
n	nano
NFT	Neurofibrillary tangles
NGF	Nerve Growth Factor
NHMS	National Health and Morbidity Survey
NMDA	N-methyl-D-aspartate
NPDepo	RIKEN Natural Products Depository
Nrf	Nuclear factor erythroid
OreR	Oregon R
PS	Presenilin
RAGE	Receptor for advanced glycation end products
REP	Rough eye phenotype
RFU	Relative Fluorescence Unit
RIKEN	Rikagaku Kenkyūjo
RLU	Relative luminescent unit
RNA-Seq	RNA-sequencing
ROS	Reactive oxygen species
SalA	Salvianolic acid A
SalB	Salvianolic acid B
SAR	structure and activity relationship
sws	*Swiss cheese*
T7 RNAP	T7 RNA polymerase
THS	Thioflavin S
THT	Thioflavin T

TNF-α	Tumor necrosis factor alpha
UAS	Upstream activator sequence
UPLC	Ultra Performance Liquid Chromatography
USM	Universiti Sains Malaysia
WST	Water-soluble tetrazolium salt

LIST OF APPENDICES

KAJIAN *IN VITRO* DAN *IN VIVO* BAGI PENGENALPASTIAN SEBATIAN YANG MENGURANGKAN NEURODEGENERASI KAITAN Aβ42

ABSTRAK

Penyakit Alzheimer (AD) adalah penyakit neurologi yang paling biasa di peringkat global. Mekanisme penyakit ini merangkumi pengumpulan plak ekstraselular senil yang terdiri daripada peptida β-amiloid (Aβ) dalam otak. Antara isomer Aβ yang dirembeskan di otak, Aβ42 adalah yang paling neurotoksik dan agresif. Walaupun terdapat banyak kajian mengenai AD, patogenesis penyakit ini masih belum diketahui. Tujuan utama kajian ini adalah untuk mengenal pasti sebatian yang boleh mengurangkan kesan negatif Aβ42. Dua kumpulan sebatian, iaitu molekul kecil daripada perpustakaan RIKEN NPDepo dan konstituen larut air dari ekstrak air Danshen (DWE) telah disaring. Pertama sekali, sebatian ini disahkan dapat mengurangkan agregasi Aβ42 berdasarkan ujian agregasi Aβ42 *in vitro* yang mengukur kuantiti pembentukan agregat Aβ42 dalam masa nyata. Dua puluh sebatian yang dapat mengurangkan agregat Aβ42 sehingga kurang daripada 70% kawalan Aβ42 yang tidak dirawat dengan sebatian dianggap sebagai penghambat agregasi Aβ42 yang berpotensi. Oleh sebab agregasi Aβ42 mengakibatkan degenerasi neuron, dua puluh perencat agregasi Aβ42 yang berpotensi ini diuji pada sel-sel neuron PC12 yang diinkubasi dengan Aβ42. Enam daripada perencat agregasi Aβ42 yang berpotensi dapat mengurangkan kematian sel PC12 yang diinkubasi dengan Aβ42 secara ketara berbanding sel kawalan PC12 yang tidak terdedah kepada Aβ42. Sebatian-sebatian ini kemudian diuji dengan menggunakan *Drosophila melanogaster*. *Drosophila melanogaster* adalah model yang baik untuk menganalisis

ciri fisiologi dan gangguan tingkah laku dalam penyakit neurodegeneratif manusia. Model AD *Drosophila* yang digunakan dalam tesis ini mengeksprasi Aβ42 manusia. *Drosophila* AD menunjukkan struktur mata yang merosot yang disebut sebagai fenotip mata kasar (REP), penurunan kemampuan lokomotif dan kematian awal. Asid salvianolik A (SalA) (100 μM), asid salvianolik B (SalB) (100 μM) dan NPD6990 (100 μM) didapati dapat memperbaiki sebahagian REP, meningkatkan pergerakan dan memanjangkan jangka hayat apabila makanan yang mengandungi sebatian ini diberi kepada *Drosophila* AD. Oleh sebab kesan penyelamatan yang efektif ini, tindak balas transkriptomik *Drosophila* AD terhadap ketiga-tiga sebatian tersebut dikaji. SalA menghalang gen biosintesis steroid seperti *Lip3* yang mungkin dapat mengurangkan pembentukan plak amiloid dalam otak. Sebaliknya, SalB meningkatkan kepekatan glutathione dalam otak yang meredakan kecederaan oksidatif yang diperburuk oleh Aβ melalui peningkatan gen yang terlibat dalam sintesis glutathione seperti *GstD8* dan *Gss2*. NPD6990 pula merencatkan tapak jalan tindak balas imun melalui gen seperti *BomS1*, *BomS2* dan *BomS3* yang mengurangkan keradangan saraf yang terlibat dalam patogenesis agregasi amiloid. Secara keseluruhan, penemuan kajian ini membuktikan SalA, SalB dan NPD6990 mempunyai potensi sebagai agen terapeutik untuk AD dan kategori gangguan otak yang lain.

IN VITRO AND *IN VIVO* STUDY ON THE IDENTIFICATION OF COMPOUNDS THAT ALLEVIATE Aβ42 ASSOCIATED NEURODEGENERATION

ABSTRACT

Alzheimer's disease (AD) is the most common type of neurological disorder globally. Its mechanism includes the distinctive aggregation of extracellular senile plaques made up of amyloid-beta (Aβ) in the brain. Among the Aβ isomers secreted in the brain, Aβ42 is the most neurotoxic and aggressive. Regardless of the immense research on AD, the full pathogenesis of this disease remains unknown. The main aim of this study was to identify compounds that are able to alleviate Aβ42's negative effects. Two groups of compounds, water soluble constituents from Danshen water extract (DWE) and small molecules from RIKEN's NPDepo library were screened. The compounds were first verified to reduce Aβ42 aggregation grounded on the *in vitro* Aβ42 aggregation assay that quantified the formation of Aβ42 aggregates in real-time. Twenty compounds that were able to reduce the amount of aggregated Aβ42 to less than 70% of Aβ42 controls that were not treated with any compounds were considered potential Aβ42 aggregation inhibitors. Since Aβ42 aggregation results in neuronal degeneration, the potential Aβ42 inhibitors were evaluated on Aβ42-incubated PC12 neuronal cells. Six of the inhibitors were able to significantly reduce cell death of Aβ42-incubated PC12 cells compared to PC12 control cells that were unexposed to Aβ42. These six compounds were then tested on the *Drosophila melanogaster*. The *Drosophila melanogaster* is an excellent model in analyzing both physiological and behavioral features of human

neurodegenerative disorders. The AD *Drosophila* model that expressed the human Aβ42 was employed. This AD *Drosophila* demonstrated deteriorated eye structures termed as the rough eye phenotype (REP), declining locomotive ability and early death. Administration of salvianolic acid A (SalA) (100 μM), salvianolic acid B (SalB) (100 μM) and NPD6990 (100 μM) were found to partially ameliorate the REP, enhanced climbing mobility and prolonged the lifespan of AD *Drosophila*. Due to these rescue effects, the transcriptomic responses of AD *Drosophila* towards the three compounds were investigated. SalA inhibited steroid biosynthesis genes such as *Lip*3 which could possibly reduce the formation of amyloid plaques in the brain. On the other hand, SalB increases glutathione concentration in the brain that combats oxidative injury exacerbated by Aβ through the upregulation of genes involved in glutathione synthesis such as *GstD8* and *Gss2*. Alternatively, NPD6990 suppressed the immune response pathway via genes such as *BomS1*, *BomS2* and *BomS3* which reduce neuroinflammation implicated in the amyloid aggregation pathogenesis. In conclusion, the findings here collectively evinced the likelihood of SalA and SalB in addition to NPD6990 as promising therapeutic properties for AD and possibly other categories of brain disorders.

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview

Ageing is the natural progression of life and is a risk factor to age-associated disorders such as dementia (Franceschi et al., 2018). According to United Nations projections, the global population of 7.35 billion as of 2019 will surpass 10 billion in 90 years (Cabrales et al., 2019). This ageing tsunami brings about complications in the form of age-related diseases.

Alzheimer's disease (AD) is the most common form of dementia and is described as a gradual and progressive decline in cognitive function. While memory decline is the most associated trait of the disease, AD patients often encounter a range of other symptoms such as behavioral deviations to motor decline, and ultimately the inability to perform the simplest tasks (Tarawneh & Holtzman, 2012). Being a multifactorial disease, there have been many hypotheses proposed on the occurrence of AD with the most recognized theory being the accumulation of amyloid-beta (Aβ) in the brain (Carmo Carreiras et al., 2013; Tan & Azzam, 2017). Of all the Aβ species synthesized in the brain, Aβ42 has been found to be the most toxic and aggressive (Phillips, 2019). However, despite the many decades of research, there has yet to be a cure for AD (Szczechowiak et al., 2019). The very limited United States Food and Drug Administration (FDA)-approved drugs targeted to AD only serve to delay the onset of AD symptoms (Godyń et al., 2016). As we move towards a global ageing population, the need for new and improved medications for AD becomes an increasing necessity.

This study's main focus is to screen for novel natural compounds that possess the ability to negate Aβ42's negative effects. On top of using *in vitro* methods to identify Aβ42 ligands, PC12 neuronal cells were also utilized as a screening platform. In addition, transgenic *Drosophila melanogaster* carrying the human Aβ42 gene was employed as the model organism to assess the compounds of interest for their effect in delaying AD. As the *Drosophila* share a similar yet simpler central nervous system to mammals, *Drosophila* research has made vital breakthroughs in the field or neuroscience (Pandey & Nichols, 2011). With a short lifespan, simple anatomy as well as genetic characteristics that further supports its role a model for neurodiseases, the *Drosophila* was the ideal organism for studying specific phenotypes required in this research (Tan & Azzam, 2017).

All in all, this investigation hopes to provide new facets to the underlying pathways of Aβ42 mechanism, and reveal novel compounds that work against Aβ42 toxicity.

1.2 Objectives

The principal aim of this thesis was to identify compounds that wereable to ameliorate Alzheimer's disease. This was subdivided into the following objectives:

(i) To identify inhibitors of Aβ42's aggregation via *in vitro* methods

(ii) To evaluate the functionality of selected compounds in a neuronal cell culture environment when exposed to Aβ42

(iii) To elucidate the compounds' ability to protect the transgenic *Drosophila melanogaster*AD model from Aβ42's ill effects

(iv) To decipher the response of *Drosophila melanogaster* expressing Aβ42 towards the compounds via transcriptomic analysis.

1.3 Outline

This thesis has seven chapters:

The current chapter presents a short overview, experimental objectives, and outlines the entire thesis.

Chapter 2 is an in-depth literature review encompassing current (at the time of writing) knowledge concerning the key casts of this research. It introduces the reader to Alzheimer disease as a problem to the public, what is presently known about its pathogenesis, and Aβ42 as an important factor in the disease mechanism. The reader is also given information about the fruit fly, *Drosophila melanogaster*, and how this model organism has contributed to Alzheimer's disease studies.

Chapters 3 to 6 are sections corresponding to individual objectives. Each of these chapters comprises of its own introduction of the topic, materials and methods, results and discussion of that particular investigation. Although later chapters utilize results from their preceding chapters, each chapter is meant to be able to stand-alone as individual mini theses.

Chapter 3 starts with an extensive *in vitro* screening of thousands of compounds, where compounds that were able to reduce the aggregation rate of Aβ42 were deemed as potential inhibitors.

In Chapter 4, these potential inhibitors were applied onto neuronal cell culture exposed to Aβ42. As not all compounds work similarly in cells as they do in *in vitro* situations, this experiment served as a secondary screening method. Compounds that were able to protect cells from Aβ42's effects were brought forward to the subsequent chapter.

Chapter 5 exploits the *D. melanogaster*, whereby transgenic *Drosophila* expressing the human Aβ42 gene were orally administered with the candidate compounds. Different behavioral aspects linked to Alzheimer's disease were scrutinized to further clarify the compounds' abilities in ameliorating the disease.

Chapter 6 comprehensively analyzes the pathways by which the compounds operate against Aβ42 using Next generation RNA-sequencing. By comparing transcripts from compound-treated transgenic *Drosophila* with the untreated samples, we would then be able to visualize which genes were implicated in the compounds' protective mechanism.

The thesis ends with Chapter 7, which includes a summary of the key findings in the preceding chapters, acknowledging limitations of the study and suggestions for amending them as well as proposals for future studies.

CHAPTER 2

LITERATURE REVIEW

2. Literature Review

2.1 The ageing tsunami– A global concern

The ageing population phenomenon is a global occurrence that has been given massive attention worldwide. Consistent with the World Population Prospects 2019 (Nations, 2019), by the year 2050, the projected ratio of those older than 65 years of age is one in six individuals, which is an increase from the recent ratio of one in eleven individuals in 2019. Similarly, the "old-age" stage, which is defined as the point when the remaining life expectancy decreases to 15 years, is progressively increasing as well (Pison, 2019). Most, if not all societies are currently undergoing this longevity revolution, with some barely stepping into the early stages while others are presently experiencing more advanced phases. Likewise, Malaysia is expected to be an ageing country by 2035, as soon as 15 % of the nation's population are categorized as senior citizens (Daim, 2019).

Unfortunately, with the surge in elderlies, there has been an increase in common ageing complications that senior citizens worldwide are experiencing. These problems are clustered into two groups: (1) physical and mental health, and (2) financial capability (He et al., 2016).

2.1.2 Ageing and dementia

Neurodegenerative disease is an umbrella term that covers a broad range of chronic or progressive brain conditions that principally affect the neurons in the brain (Ropper et al., 2014). On the other hand, dementia is a symptom of certain

neurodegenerative diseases and is most commonly associated with the deterioration of intellectual aptitudes that is severe enough to disrupt a person's ability to perform daily activities (Ropper et al., 2014). Such decline is occasionally preceded by the loss of emotional control, personality changes, or motivation (Sadock & Sadock, 2011).

The global cost of dementia in 2019 was estimated to be USD 800 billion per annum which was predicted to increase up to USD 2 trillion by 2030 (Chan et al., 2019). The Alzheimer's Disease International (ADI) testified that 46.8 million people globally were affected by dementia, with the total number doubling every 20 years to an estimate total of 74.7 million by the year 2030 (Patterson, 2018). Focusing on Malaysia's elderly population, our nation has an approximate number of 123,000 individuals with dementia which amounts to a total healthcare cost of USD 175 million per year (Prince, 2015). The National Health and Morbidity Survey (NHMS) reported that a total of 8.5 % of Malaysian elderlies over the age of 60 were experiencing dementia (Mustaming et al., 2018).

The highest risk factor for dementia is increasing age. After the age of 65 years, the prevalence and incidence of dementia doubles in every five to six years and around 30 % of individuals aged above 85 years might be affected by dementia (Patterson, 2018). Furthermore, approximately 80 % of reported dementia cases were elderlies aged above 75 years (Fratiglioni & Qiu, 2011). This is a grave problem for the public well-being and health policy development as the oldest senior citizens (for instance the octogenarians, nonagenarians, and centenarians) are the fastest increasing sector of every population. Thus, dementia is and will be a huge burden to the ageing tsunami. However, despite occurring more often with age, it is crucial to understand that dementia is not a part of normal ageing and it is not the

inevitable fate of the elderly. In fact, there have even been reports of “young” onset dementia (occurring prior to the age of 65 years) which makes up 9 % of total records worldwide (Cahill, 2019).

2.2 Alzheimer’s Disease (AD) – The plight of forgetfulness

AD was first discovered by Dr Alois Alzheimer while examining 50 year old Auguste Deter (Alzheimer, 1906). Dr Alzheimer described that Deter was suffering from short-term memory, confusion and disorientation. His autopsy of Deter’s brain identified neurofibrillary tangles, senile plaques and brain atrophy (Cipriani et al., 2011). These brain abnormalities would later be recognized as attributes of AD.

As the most widespread form of dementia worldwide, AD accounts for 40 % to 80 % of documented dementia cases (Nussbaum & Ellis, 2003). Hitting closer to home, approximately 50,000 Malaysians were living with the disorder (Habash et al., 2013). Likewise with dementia, although age is the single paramount risk factor for developing the disease, it is not the direct cause of AD (Dewachter et al., 2000). Individuals at 70 years and above have a 10 % risk of developing AD which increases to 45 % for those aged above 85 years (Bird, 2008). While the disorder is frequently linked with cognition decline, patients often experience a range of other symptoms such as motor dysfunctions and behaviour changes. Regardless of discrepancies in patient symptoms, molecular analysis showed that the genetic makeup of the disease is conserved (Theuns & Van Broeckhoven, 2000). Conversely, environmental factors associated with AD include sleep deprivation, exposure to environmental insults or stressors such as psychological stress, environmental toxins, hypothermia, anesthesia, brain trauma and injury, starvation and glucose hypometabolism (Killin et al., 2016; Wainaina et al., 2014).

2.3 Genetics of AD

There are two classifications of genes that determine the occurrence of a disease: (1) risk genes and (2) deterministic genes. Both types were identified in AD pathogenesis. Risk genes raises the likelihood of developing a disease while triggering the emergence of symptoms but does not guarantee the manifestation of the disease (Karch & Goate, 2015). On the other hand, deterministic genes are genes that directly cause the disease, therefore guaranteeing the development of the disease when inherited. These genes accounts for 5 % of AD cases whereby individuals experience familial early-onset forms of AD (Reitz & Mayeux, 2014). In contrast, the majority of AD patients are diagnosed with late-onset disease (Zou et al., 2014).

There are multiple motives for studying the genetic etiology of AD and its connection to AD neuropathology: 1) Unravelling the underlying genetics leads to a deeper understanding of the disease pathophysiology. 2) Distinct from most environmental risk factors, genetic risk factors may be modifiable. 3) Genetic risk factors are potential drug targets and thus allow for the production of personalized disease treatments. 4) Genetic risk factors can be utilized as biomarkers to detect at risk populations for early disease prevention.

2.3.1 Early onset familial Alzheimer's disease (EOFAD)

AD associated to genetic causes is known as early onset familial Alzheimer's disease (EOFAD). Fortunately, while EOFAD is more severe and progresses rapidly, the manifestation of EOFAD is relatively rare; making up about 5 % of all AD cases (Bagyinszky et al., 2014). EOFAD is inherited through autosomal-dominance and has a large multi-generational lineage that facilitates genetic analysis. Furthermore, the occurrence of symptoms prior to the age of 65 allows for early detection (Bird,

2008). The three main genes associated with EOFAD are part of the amyloid pathology: amyloid-beta precursor protein (*APP*) (Goate et al., 1991), presenilin 1 (*PSEN1*) (Sherrington et al., 1996) and presenilin 2 (*PSEN2*) (Schneider et al., 2014). These genes affect the processing or production of Aβ, the main component of amyloid plaques seen in Alzheimer's disease patients (Bagyinszky et al., 2014). Mutations in both *APP* and *PSEN2* account for less than 20 % of the total EOFAD cases, while *PSEN1* mutations were discovered in 80 % of EOFAD patients (Hutton & Hardy, 1997).

2.3.1(a) Amyloid precursor protein (APP)

The amyloid precursor protein (*APP*) gene encodes for a fundamental membrane-related type-1 transmembrane protein that is made up of a sizable extracellular amino terminal region and a minor intracellular cytoplasmic region (Nilsberth et al., 2001). The extracellular region covers a cysteine-rich sub-domain adjacent to the amino terminal, an acidic sub-domain and another two sub-domains, of which one has alleged neuroprotective properties (Nilsberth et al., 2001). *APP* contains 19 exons across 290 kb which encodes for a protein approximately 695-770 amino acid long (Zheng & Koo, 2006). The Aβ protein is encoded by exons 16 and 17 (Yoshikai et al., 1990). *APP* is found on chromosome 21 in humans. Indeed, Down syndrome patients carrying an extra chromosome 21 also demonstrated AD-like symptoms (Wisniewski et al., 1985). The *APP* gene expression typically takes place in cells and tissues of the neurons, glia and endothelia in the brain. No less than three major protein conformers, APP695, APP751 and APP770 are cut alternatively from the *APP* pre-mRNA (Tomiyama et al., 2008). The protein, APP is located in the cell membrane, Golgi compartments and endoplasmic reticulum (Schellenberg et al.,

1992). During normal neuronal function, APP behaves as a G-protein-coupled receptor that assists in synaptic plasticity and transmission as well as cell adhesion (Nilsberth et al., 2001).

As of now, there are 33 different *APP* mutations recognized in AD patients: 1 deletion, 9 duplications and 23 missense mutations (Hutton & Hardy, 1997) (Known APP mutations are shown in Figure 2.1). As the mutations are dominantly inherited, they are found close to or within the β-secretase and γ-secretase splice sites in exons 16 and 17 of the *APP*; hence, these mutations impact the resulting protein's proteolytic processing, C-terminal fragment stability and aggregation of APP C-terminal fragments and Aβ aggregation (Kovacs et al., 1996). Table 2.1 shows the different *APP* mutations. While clinical symptoms of mutation carriers differs, all *APP* mutations have modified APP proteolytic processing that either brings about an upsurge in total Aβ production or Aβ42 compared to wild-type APP which leads to an overall increase in Aβ42/Aβ40 ratio (Nilsberth et al., 2001).

Table 2.1: List of mutations near to or within the Aβ region of the *APP*

Type/location of mutation	Mutation name	Reference
Double mutation at the N-terminal of the Aβ region near to the β-secretase site	*KM670/671NL* (Swedish mutation)	(Fukumori et al., 2010)
C-terminal of the Aβ region close to the γ-secretase cleavage site	*T714A* (Iranian mutation) *T714I* (Austrian mutation) *V715M* (French mutation) *V715I* (German mutation) *I716V* (Florida mutation) *V717I* (London mutation) *K724N* (Belgian mutation) *L723P* (Australian mutation) *I716F* (Iberian mutation) *V717F* (Indiana mutation)	(Kim et al., 1997) (Thinakaran et al., 1996) (Janssen et al., 2003) (Vetrivel et al., 2006) (Van Cauwenberghe et al., 2016) (Goate et al., 1991) (Sherrington et al., 1996) (Shen et al., 1997) (Guerreiro et al., 2010) (Murrell et al., 1991)
Point mutations located within the Aβ coding domain	*A692G* (Flemish mutation) *E693K* (Italian mutation) *E693Q* (Dutch mutation) *E693G* (Arctic mutation) *D694N* (Iowa mutation)	(Wong et al., 1997) Cruts & Van Broeckhoven, 1998) (Farrer et al., 1990) (Myers et al., 1996) (Kayden et al., 1985)
Deletion mutation at *APP* 693	Δ*E693* (Osaka mutation)	(Tomiyama et al., 2008)

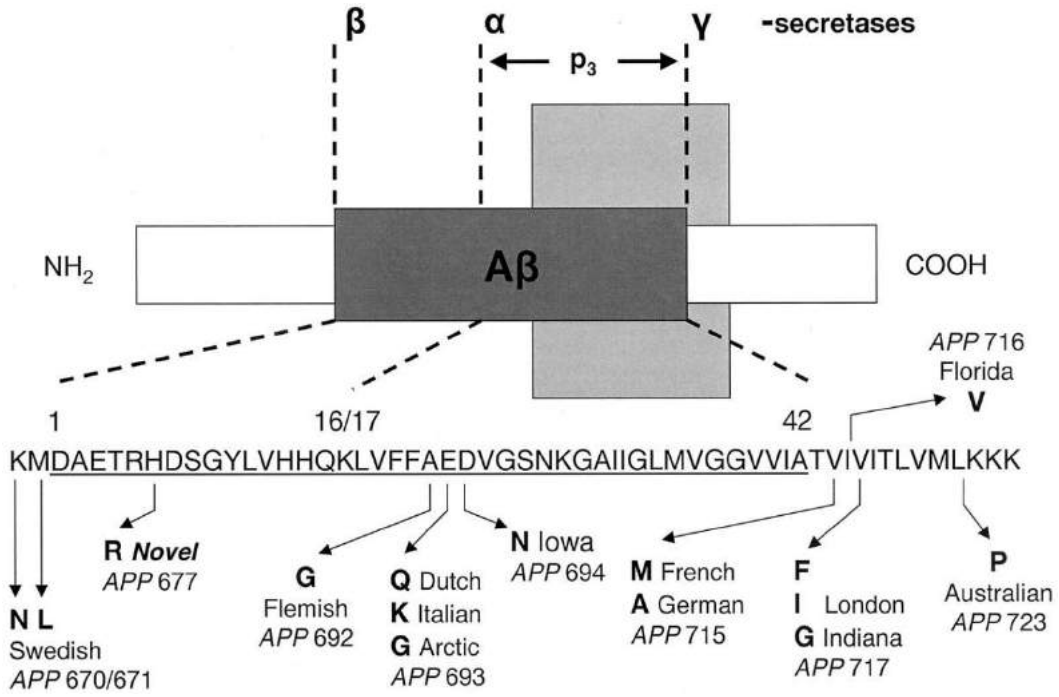

Figure 2.1: Pathogenic mutations within the Aβ region of the APP. Adapted from (Janssen et al., 2003).

2.3.1(b) Presenilin (PS)

Besides APP gene mutations, changes in presenilin genes are also implicated in AD pathogenesis. Presenilins (PS) that encode the *PSEN* genes are transmembrane region proteins which make up part of the catalytic subunits of the γ-secretase intramembrane protease complex (Iwatsubo, 2004). The human *PSEN1* and *PSEN2* genes are located on chromosome 14 (Schellenberg et al., 1992) and chromosome 1 (Levy-Lahad et al., 1995), correspondingly. The PS-1 protein is a 42- to 43 kDa polypeptide (Thinakaran et al., 1996) while the PS-2 protein has a size of 53-55 kDa (Kim et al., 1997). The presenilin proteins are spliced by unknown proteases in the α-helical sequence of the cytoplasm loops which leads to a protein with a larger N-terminal portion and a minor C-terminal fragment. Consequently, the two fragments are fused to form a functional protein (Hutton & Hardy, 1997). The *PSEN1* gene has a significant role in intra-membrane mediation and encodes a serpentine protein which is generally found in the Golgi apparatus, endoplasmic reticulum and nuclear envelope (Fukumori et al., 2010; Kovacs et al., 1996). RNA transcripts of *PSEN1* can be found in the human heart, placenta, pancreas, kidney, skeletal muscle and brain (Kovacs et al., 1996). In contrast, *PSEN2*'s exact role is still a mystery; however, it is presumed that proteins of *PSEN2* and *PSEN1* work together as constituents for γ- secretase (Vetrivel et al., 2006). As γ-secretase is involved in APP splicing that generate Aβ40 and Aβ42 fragments, changes in either presenilins will cause the proliferation of secreted Aβ42 or reduce the concentrations of Aβ40 (Cruts & Van Broeckhoven, 1998).

2.3.2 Late onset Alzheimer's disease (LOAD)

There is a strong genetic basis for late-onset Alzheimer's disease (LOAD); thus far, 22 genes/loci that affect the risk of LOAD have been identified. LOAD inheriting families have low kin survivability at the particular onset period as well as being low in genetic data of the parents. In a study assessing 70 families with one or more AD patients, the offspring of AD patients had an expected lifetime risk of 53 % in EOAD families versus LOAD family kin that had an 86 % risk (Farrer et al., 1990). This outcome reinforced that EOAD is autosomal dominantly transferred to the kin while LOAD had a heterogeneous transmission with a combination of genetic, environmental and lifestyle contributions (Zou et al., 2014).

Out of all LOAD cases, 15 % of patients carry an Apolipoprotein E (*APOE*) allele (Myers et al., 1996). *APOE* is located at chromosomal locus 19q13.2 and codes for a glycoprotein the size of 299 amino acids. It is produced in the monocytes, resident macrophages, liver, and brain of humans (Kayden et al., 1985). APOE protein acts as a transport for lipids and also functions in lipolytic enzyme activation, neuronal growth, immune-regulation and repair of tissues (Van Cauwenberghe et al., 2016). In addition, APOE also contributes to the re-modeling and repairing neurons through various pathways such as the anti-oxidation, oestrogen interaction and synaptodendritic proteins regulation (Khanahmadi et al., 2015). Mouse studies have shown that APOE is involved in the production of neuritic and cerebrovascular plaques (Holtzman et al., 2000). APOE also plays a role in Aβ homeostasis in the brain by mediating both the accumulation and removal of Aβ (Verghese et al., 2013).

Interestingly, certain Nigerian populations do not show any association between *APOE4* and AD age-of-onset as seen in other populations (Gureje et al., 2006). This suggested that there is incomplete penetrance of *APOE* and also the

involvement of other genes in LOAD. The human brain possesses three *APOE* alleles that are located at the same gene locus (ε2, ε3 and ε4) encoding for isoforms *APOE2*, *APOE3* and *APOE4*, respectively (Roses, 1996). These isoforms are structurally distinguishable by two amino acid substitutions at residues 112 and 158: APOE3 carries Cys112 and Arg158; in APOE2, cysteine substitutes Arg158, while arginine substitutes Cys112 in APOE4 (McKeon-O'Malley & Tanzi, 2001). These differences in amino acid positioning affect APOE function by altering the structure and charge of the respective protein (Verghese et al., 2013).

Carriers of the ε4 allele tend to inherit both early and late onset AD (Corder et al., 1993). There is an expected threefold risk of developing AD for ε34 genotype heterozygous carriers while homozygous carriers of ε4 allele have a 15-fold risk (Farrer et al., 1997; Saunders et al., 1993). Additionally, ε4 allele carriers have an amplified risk from two to five fold with an earlier onset age of 7.7 years compared to homozygous ε3 allele carriers (Corder et al., 1993). On the other hand, carriers of the ε2 allele had a delayed onset age of AD symptoms (Corder et al., 1993). Each APOE allele affects the concentration of Aβ42 secreted in the brain with ε4 having the highest concentration of Aβ42 produced, followed by ε3 and finally ε2 (Castellano et al., 2011). Homozygous carriers of the ε4 allele generally develop AD by 80 years old (Corder et al., 1993).

2.4 Amyloid pathway hypothesis

The construction of the amyloid pathway hypothesis, otherwise known as the amyloid cascade hypothesis, was compiled from various data founded from the chronological events – (1) the earliest record in 1906 of senile plaques and neurofibrillary tangles (NFTs) by Dr Alois Alzheimer from his autopsy of an AD

patient's brain (Alzheimer, 1906), (2) the successful extraction of Aβ from senile plaques in 1984 (Glenner & Wong, 1984), (3) sequencing of the *APP* gene in 1987 (Kang et al., 1987) (4) discovery of *APP* autosomal dominant mutations (Goate et al., 1991). (5) proposal of the amyloid pathway hypothesis in 1992 (Hardy & Higgins, 1992) that was reappraised in 2006 (Hardy, 2006). This theory suggested that the manifestation of AD was because of two types of genetic mutations: LOAD and EOFAD.

2.4.1 Aβ production, oligomerization and fibrillization

Total cellular APP's half-life is short of about 30 to 60 mins (Storey et al., 1999) and its post-translational processing consists of two pathways (Figure 2.2). In the non-amyloidogenic pathway, α-secretase begins by cleaving within the Aβ sequence (between residues Lys687 to Leu688) which causes Aβ to be inactive and non-toxic peptides are produced (McKeon-O'Malley & Tanzi, 2001). Splicing by γ-secretase at the residual C-terminal extracellularly secretes the non-toxic P3 peptide whereas the APP intracellular domain (AICD) is retained in the cell (Hardy, 1997). Conversely, the amyloidogenic pathway involves the cleavage of APP by β-secretase directly at Aβ's N-terminal (between residues Met671–Asp672) (Hardy, 1997). Additional splicing by γ-secretase at Aβ's C-terminal yields a functional Aβ peptide that is excreted out of the cell (Prüßing et al., 2013). Due to γ-secretase's heterogeneous splicing nature, various lengths of Aβ species are secreted (O'Brien & Wong, 2011).

Aβ's self-aggregation is affected by its sterics, secondary structure propensity, charge, and hydrophobicity (Senguen et al., 2011). Due to Aβ's self-assembly features, Aβ's soluble monomers can have various mis-folding

arrangements that produce different concentrations of protein aggregates either under or near physiological environments. In addition, specific mis-folded oligomers, termed as “seeds”, are able to induce other Aβ to mimic the mis-folded oligomeric structure. This causes a chain reaction similar to a prion infection (Haass & Selkoe, 2007). Aβ’s aggregation kinetics is reliant on the C-terminal residues, therefore, Aβ42 experiences a more rapid fibrillization compared to Aβ40. In fact, the presence of Aβ42 peptides hastens Aβ40 fibrillization (Jarrett et al., 1993). This supports findings wherein familial EOFAD brains have elevated Aβ42 to Aβ40 ratio levels compared to healthy brains of the same age range (Scheuner et al., 1996).

Generally, Aβ fibril formation starts with a lag phase, whereby a thermodynamically stable nucleus is required to aggregate and is succeeded by a rapid elongation phase (Harper & Lansbury Jr, 1997). This results in the formation of large insoluble amyloid fibrils that are anti-parallel and cross-β-sheet in structure (Lee & Ham, 2011). However, the exact Aβ species that is responsible for neurotoxicity to the AD brain has yet to be determined.

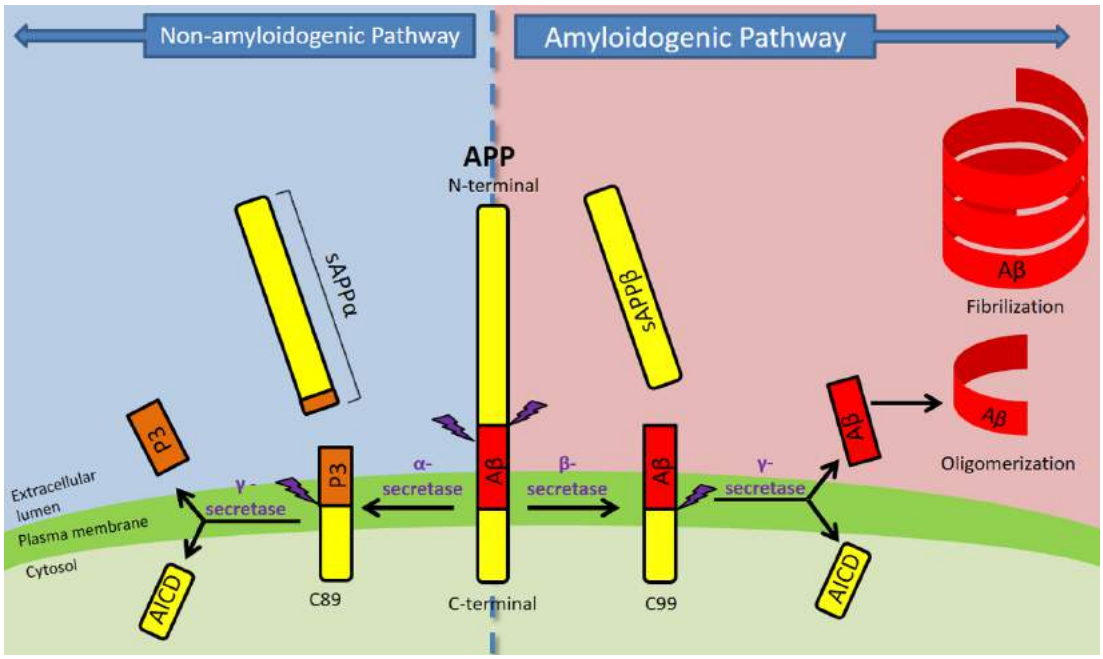

Figure 2.2: APP proteolysis. The left portion depicts the non-amyloidogenic pathway wherein APP is first cut by α-secretase and consecutively by γ-secretase to produce the non-toxic P3 peptide and AICD fragment. On the right, the amyloidogenic pathway consists of β-secretase splicing of APP and consecutively by γ-secretase to yield Aβ peptides. Aβ peptides undergo oligomerization, fibrilize and finally produce insoluble plaques. Adapted from (Tan & Azzam, 2017).

2.4.1(a) Aβ proteins – its normal physiology and toxic forms

The Aβ protein was first acknowledged as a potential biomarker of AD when it was discovered as "a novel cerebrovascular amyloid protein" in 1984 (Glenner & Wong, 1984). The following year, this 4.2 kDa protein was recognized as a main component of senile plaques in AD brains (Wong et al., 1985). The size of Aβ varies between 39 to 43 amino acid residues, all of which are produced in the AD brain (Hamley, 2012).

Aβ40 is the most prominent conformer followed by Aβ42 in both normal and AD human brains (Seubert et al., 1992). Compared to Aβ40, Aβ42 has an extra isoleucine and an extra alanine at the C-terminal. Despite the high similarity in sequence identity, Aβ40 is lower in amyloidogenicity but higher in solubility than

Aβ42 (Snyder et al., 1994). The early structures and stabilities of Aβ42 and Aβ40 are distinctive from each other with Aβ40 occurring as monomers while Aβ42 exists as equal amounts of trimer/tetramer and monomers (Sgourakis et al., 2007). Aβ40 and Aβ42 undergo different fibrillization pathways, whereby Aβ42 produce pentameric/hexameric paranuclei while Aβ40 experiences monomer accumulation (Bernstein et al., 2009). Aβ42's higher hydrophobicity is most likely the reason why it is able to integrate into the lipid bilayer which thus leads to cell injury (Butterfield et al., 2013). In addition, Aβ42's more structured C-terminal may aid its tendency to aggregate (Lim et al., 2007). Aβ40 has been shown to inhibit aggregation of Aβ42 by preferentially binding onto Aβ42 proto-fibrils (Yan & Wang, 2007). Changes in Aβ42 to Aβ40 ratio leads to different neurotoxicity intensities (Kuperstein et al., 2010). Aβ40 is primarily linked to cerebral amyloid angiopathy (DeSimone et al., 2017), while Aβ42 is the main constituent in amyloid plaques and has a higher correspondence with AD pathology (Roses, 1998).

While Aβ has been implicated in AD pathology, the protein exists in normal brains albeit at low concentrations (Cirrito et al., 2003). From this, it is highly likely that Aβ is required for normal physiological function in the brain specifically in moderating synaptic activity and neural survivability (Pearson & Peers, 2006). Aβ's nature to be either ameliorating or aggravating depends on both its relative concentration and also the cellular environment it resides in (Parihar & Brewer, 2010). Increased Aβ concentrations (nM to μM) resulted in neurotoxicity and neuronal death (Jellinger, 2006). Contrariwise, low concentrations of Aβ, in pM, served as trophic signals and as a mediator of synaptic activity in addition to modulating neuron cell viability (Plant et al., 2003). At this low amount, Aβ was found to act as antioxidants that latched onto redox-active metals such as zinc and

iron. This binding was shown to inhibit these metals from redox cycling with other ligands (Atwood et al., 2003).

Aβ's concentrations in the brain is affected by an equilibrium of several considerations: (1) the modulation of APP cleavage and Aβ construction, (2) Aβ removal and facilitation of Aβ through the blood-brain barrier, (3) proteolytic degradation of Aβ, (4) oligomerization of Aβ and (5) the aptitude of Aβ to latch onto and sequester other Aβ proteins, which consecutively governs Aβ aggregation and clearance. Accordingly, the disruption of the homeostatic state between Aβ secretion and its elimination will possibly cause the onset of AD (Bates et al., 2009). Besides that, modifications to Aβ's structure from post-translational dysregulations might also hasten AD's pathological events (Parihar & Brewer, 2010).

The discovery of amyloid deposits in senile plaques of all AD human brains prompted the hypothesis of amyloid cascade whereby AD onset is caused by the aggregation of soluble Aβ into insoluble fibrils. Fresh Aβ is non-toxic. However, amyloid fibrils were found to cause neurotoxicity by amplifying the number of both the action potentials and depolarisation of the membrane in cell cultured neurons (Howlett et al., 1995; Kowall et al., 1991; Lorenzo & Yankner, 1996). Furthermore, rats with compromised synaptic transmission demonstrated cognitive or memory dysfunction and death of neurons when injected with amyloid fibrils into the rat dorsal dentate gyrus (Stephan et al., 2001). Fibrillar Aβ has also been proven to bind to various cell surface proteins, as well as the receptor for advanced glycation end products (RAGE) complex and APP. These bindings cause a surge in free radical production and oxidative stress (Verdier & Penke, 2004). Likewise, the binding of Aβ fibrils to the α-7 nicotinic receptor modulate N-methyl-D-aspartate (NMDA) receptor results in defects towards cellular metabolism including the loss of synaptic

function that is implicated in symptomatic AD (Snyder et al., 2005). Some studies exhibited that the progression and severity of AD is reliant on the concentration of aggregated insoluble Aβ fibrils (Lorenzo & Yankner, 1996; Meyer-Luehmann et al., 2008). A distinguishing feature of Aβ fibrils is that the same type of fibril can have various morphologies based on the aggregation environments (Petkova et al., 2002; Xu et al., 2014). Such manifestations are termed as Aβ fibrils polymorphism which greatly influences the neurotoxicity of the fibrils (Petkova et al., 2002).

On the other hand, amyloid plaques are the abnormal, proteinaceous, fibrous deposits with diameters between 7 to 10 nm and a β-sheet secondary formation (Sunde et al., 1997). These plaques are largely made up of Aβ proteins and Aβ-related proteins such as vitronectin, apolipoprotein J, APOE, α1-antichymotrypsin and other non-Aβ constituents (Yamaguchi, 1999). There are two types of amyloid plaques often seen in AD – diffuse plaques and dense core plaques (Thal et al., 2006).

Diffuse plaques are found at first in the neuropil and are weakly stained by Thioflavin S (THS) and amyloidophilic dyes such as Congo red (Teplow et al., 2012). It is understood that diffuse plaques occur prior to senile plaques (Gyure et al., 2001). In their early formation, diffuse plaques are amorphous instead of fibrils (Yamaguchi, 1999). At their later stages, production of low amounts of fibrillary Aβ are detected between cell processes (Yamaguchi, 1999). Compared to diffuse plaques, senile plaques have a dense reticular amyloid core that is rich in long Aβ proteins. Since senile plaques are denser with abundant fibrils, they are intensely stained positive with THS and Congo red (Teplow et al., 2012).

It has been revealed that microglia associate with amyloid plaques (Mandrekar-Colucci & Landreth, 2010). Aβ, either in the protomeric or oligomeric stages, may

be the main factor prompting the activation of microglia which causes an abnormally vigorous neuroinflammatory reaction (Garden & Möller, 2006). The gliosis and neuroinflammation derived from the aggregation of Aβ protein is itself neurotoxic (Leyns & Holtzman, 2017). Amyloid plaques in the brains of AD patients are generally surrounded by activated microglia, which implies that the cytokines and cytotoxic molecules secreted by microglia may function in the disease pathogenesis (Jung et al., 2015).

2.5 Animal models of AD

It is undisputable that human genetic research has enhanced our comprehension on genes related to neurodegeneration. Nevertheless, investigations on human subjects are limited by ethical and technical restrictions. As such, we look to animals to mimic human diseases. AD models comprise of the fruitfly (*Drosophila melanogaster),* mouse (*Mus musculus*), zebrafish (*Danio rerio*), and nematode (*Caenorhabditis elegans*)*;* each emulating different aspects of AD (Table 2.2).

Table 2.2: Evaluation of common animal models. Adapted from (Tan & Azzam, 2017).

Organism	Advantages	Disadvantages
Mus musculus (Mouse)	• Mammal brain anatomy similar to humans • Sophisticated behavioural analysis • Histopathology testing accessible • Targeted gene replacement available	• Relatively expensive • Long life-cycle • Complex gene manipulation procedures • Ethical considerations • Laborious • Inefficient
Caenorhabditis elegans (Roundworm)	• Relatively inexpensive • Short life cycle • Small size • Large population • Genomics known	• Poor illustration of some signalling pathways • Retains fewer gene homologs in mammals • Lacking in many vital organs available in humans • No male/female sexual system • Brain is not centralized • Challenging to evaluate behavioural abnormalities
Danio rerio (Zebrafish)	• Simple vertebrate structure • Transparency permits easy observation • External embryos • Excellent organogenesis model	• Relatively expensive • Long life cycle • Genetics and genomics studies still developing

2.5.1 *Drosophila melanogaster:* A comprehensive model

The *Drosophila melanogaster,* otherwise known as the fruit fly, has contributed tremendously to genetics and neuroresearch. Presently, there are *Drosophila* models for most neurodiseases including AD, Huntington's disease, motor-neural disease, transthyretin-related amyloidotic polyneuropathy and polyQ-associated expansion conditions (Moloney et al., 2010).

Thomas Hunt Morgan first introduced *Drosophila* into the field of genetics in 1908. It was Thomas' suspicions in Gregor Mendel's laws of inheritance that led Thomas to dabble in *Drosophila* research where he ultimately discovered the theory of genes as the carrier of hereditary information (Morgan, 1910).

The *Drosophila*'s genome size of about 175 Mb with approximately 13,600 genes (Ellis et al., 2014) is minuscule in contrast to the human genome of approximately 50,000 genes (Alles et al., 2019). Out of the 287 documented human disease genes, 197 (69%) have a *Drosophila* homolog (St Johnston, 2002). Moreover, *Drosophila* have fewer genetic redundancy than vertebrate models, making gene characterization less complex.

There are many assets that make *Drosophila* such an attractive organism to observe. The fruit fly has a short lifespan and is regarded as a four-in-one model due to its life history that comprises of distinguishable morphological phases: the embryo, larva, pupa and adult, each providing distinct modelling purposes (Pandey & Nichols, 2011). Moreover, care and housekeeping requires little equipment with low overall cost.

Drosophila's simple anatomy and genetic features benefit it in its role as an exemplary disease model. There is neither meiotic recombination nor synaptonemal complex in male *Drosophila* (Orr-Weaver, 1995; St Johnston, 2002). Therefore,

recombinant manipulation is concentrated only on females. Differentiating males and females can be efficiently done under the light microscope due to their obvious anatomical distinctions. In addition, a single female lays hundreds of offspring within a day, making it easy for large scale genetic screening experiments. *Drosophila* have four pairs of chromosomes that can be easily observed as huge polytene chromosomes whereby denser areas represents transcription activity. Furthermore, the use of balancer genes that function to halt heterozygous recombination has also assisted *Drosophila* studies (Bourguet et al., 2003).

Besides that, *Drosophila*'s brain is a similar albeit less complex central nervous system compared to vertebrates. Both systems comprise of neurons and secondary glia with identical neurotransmitters that are secured by a blood-brain barrier. This proves that the rudimentary principles of the neural system are well-maintained from invertebrates to vertebrates. The *Drosophila* model also displays cellular processes that are required in neurodegeneration such as oxidative stress. Complicated age-dependent behaviours including memory and locomotor capability can also be observed in the *Drosophila* (McGurk et al., 2015).

To develop the *Drosophila* into an AD model, researchers integrated the UAS-GAL4 system into the fly (Figure 2.3). The yeast-extracted transcription factor GAL4 is linked to a tissue-specific promoter gene that is already present in the *Drosophila*. Conversely, the yeast galactose upstream activator sequence (UAS) which is activated by GAL4, is attached upstream from the human disease gene (Fischer et al., 1988). To allow for various genetic recombinations, UAS and its partner gene are inserted into a *Drosophila* line that lacks the GAL4 sequence. Mating of these two lines will generate offspring that express the human disease protein in desired tissues. GAL4 driver lines that are commonly used in neurotoxicity