Drosophila melanogaster: Deciphering Alzheimer's Disease

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Abstract

Alzheimer's disease (AD) is the most widespread neurodegenerative disorder worldwide. Its pathogenesis involves two hallmarks: aggregation of amyloid beta (Aβ) and occurrence of neurofibrillary tangles (NFTs). The mechanism behind the disease is still unknown. This has prompted the use of animal models to mirror the disease. The fruit fly, *Drosophila melanogaster* has garnered considerable attention as an organism to recapitulate human disorders. With the ability to monopolise a multitude of traditional and novel genetic tools, *Drosophila* is ideal for studying not only cellular aspects but also physiological and behavioural traits of human neurodegenerative diseases. Here, we discuss the use of the *Drosophila* model in understanding AD pathology and the insights gained in discovering drug therapies for AD.

Keywords: Alzheimer, Drosophila, model organism

Introduction

Ageing is a natural progression and as we age, our body sustains more damage than it can repair which consequently pose a risk toward age-related diseases (1). Currently, about 12% (around 800 million) of the world population are aged 60 and above, and it is expected to reach 21% (2 billion) by 2050 (2). In 2016, the cost of care for dementia is estimated to be $236 billion (3).

The phrase "neurodegenerative diseases" encompasses diseases pertaining to the brain's neurons; their structure and function. They are usually asymptomatic throughout the nervous system development and maturation, therefore are generally at adult onset. Each disease pathogenesis involves the slow decline of neuronal populations specific to the disease. So far, mechanisms behind the development of these diseases are still unclear; hence there is no cure or prevention for either disease.

Alzheimer's disease (AD) is deemed the leading bane to the ageing population. It is a progressive neurodegeneration that was first characterised by Alois Alzheimer (4). While AD is commonly associated with cognitive loss, patients often experience symptoms ranging from motor deterioration to behavioural changes. Despite inconsistencies in patient symptoms, molecular analysis revealed that the genetic makeup of the disease is maintained. In 2015, 46.8 million people globally were reported to be living with the illness (5). In Malaysia, approximately 50,000 people have developed the disorder (6). Individuals above the age of 70 have a 10% risk of developing AD which increases to 45% when aged above 85 years (7).

There are many hypotheses surrounding the mechanisms of AD. The most established theories include amyloid aggregation and tauopathy. Post mortem analysis of AD patients' brains showed trademark histopathological lesions—amyloid plaques and neurofibrillary tangles (NFTs). The amyloid plaques or senile plaques mainly comprise of folded amyloid beta peptides. The build-up of amyloid beta peptides is a hallmark phenomenon found explicitly in AD pathogenesis. These peptides are by-products of the amyloid precursor protein (APP) after several

proteolytic processes. Likewise, neurofibrillary tangles are composed of hyperphosphorylated Tau proteins (8, 9).

AD linked to genetic causes is characterised as early onset familial Alzheimer's disease (EOFAD). The manifestation of EOFAD is relatively rare, making up only 5% of total AD cases. The symptoms emerge before the age of 65 and is inherited through autosomal-dominance (10). EOFAD has a large multi-generational lineage that facilitates genetic analysis (7). In contrast, late onset Alzheimer's disease (LOAD) families have low survivability of kin at the exact onset age besides having shortage in parental genetic data.

It is undeniable that human genetic research has improved the understanding of genes linked to neurodegeneration. However, examination on human subjects is obstructed by ethical and technical constraints. Thus, we turn to animals to recapitulate human diseases. AD models include the fruit fly (*Drosophila melanogaster)*, mouse (*Mus musculus*), zebrafish (*Danio rerio)*, and nematode (*Caenorhabditis elegans);* each mirroring differing aspects of AD (Table 1).

The *Drosophila melanogaster* model has made its mark in science as a great tool to study human disorders. There are currently *Drosophila* models for most neurodegenerative diseases including Huntington's disease, a range of polyQ-associated expansion disorders, transthyretin-related amyloidotic polyneuropathy, motor neuron disease, and finally AD (11). This review will shed light on the development of *Drosophila* as an AD model and its contribution as a drug discovery tool for the disease.

The amyloid pathway hypothesis

Amyloid beta (Aβ) peptide build-up is a phenomenon found explicitly in AD pathogenesis. Neuritic plaques are made up primarily of Aβ peptides derived from APP located on chromosome 21 in humans. True enough, Down syndrome patients containing an extra chromosome 21 express AD-like symptoms. APP contains 19 exons across 290 kilobases which encodes for a protein approximately 695–770 amino acid long. The Aβ protein is encoded by exons 16 and 17 (12).

APP's half-life is short and its post-translational processing comprises of two pathways (Figure 1). In the non-amyloidogenic pathway, alpha-secretase starts by splicing within the Aβ region (between residues 687 and 688). This renders the Aβ gene inactive and non-toxic fragments are formed. Cleavage by gamma-secretase at the remaining C-terminal extracellularly excretes a P3 fragment while the APP intracellular domain (AICD) is maintained in the cell (13).

In the amyloidogenic pathway, beta-secretase cuts APP right at the N-terminal of the Aβ region (between residues 671 and 672) (13). Further cleaving by gamma-secretase at the C-terminal of the Aβ gene produces an Aβ peptide which is excreted out. The peptide binds to other Aβ peptides and accumulate into fibrils.

Table 1. Comparison of common animal models

| Organism	Advantages	Disadvantages
Mus musculus (Mouse)	• Similar brain anatomy to humans • Sophisticated behavioural testing • Histopathology testing available • Targeted gene replacement possible	• Relatively expensive • Long life cycle • Complicated gene manipulation process • Ethical considerations • Laborious • Inefficient
Caenorhabditis elegans (Roundworm)	• Relatively inexpensive • Short life cycle • Small size • Large population • Genomics known	• Poor representation of some signalling pathways • Possess fewer gene homologs in mammals • Many key organs available in humans are absent • Male/female sexual system is absent • Different brain anatomy from humans; no centralised brain • Difficult to assess behavioural abnormalities • Difficult to assess plaque staging
Danio rerio (Zebrafish)	• Simple vertebrate structure • Transparency allows easy observation • External embryos • Good organogenesis model	• Relatively expensive • Long life cycle • Genetics and genomics research in progress
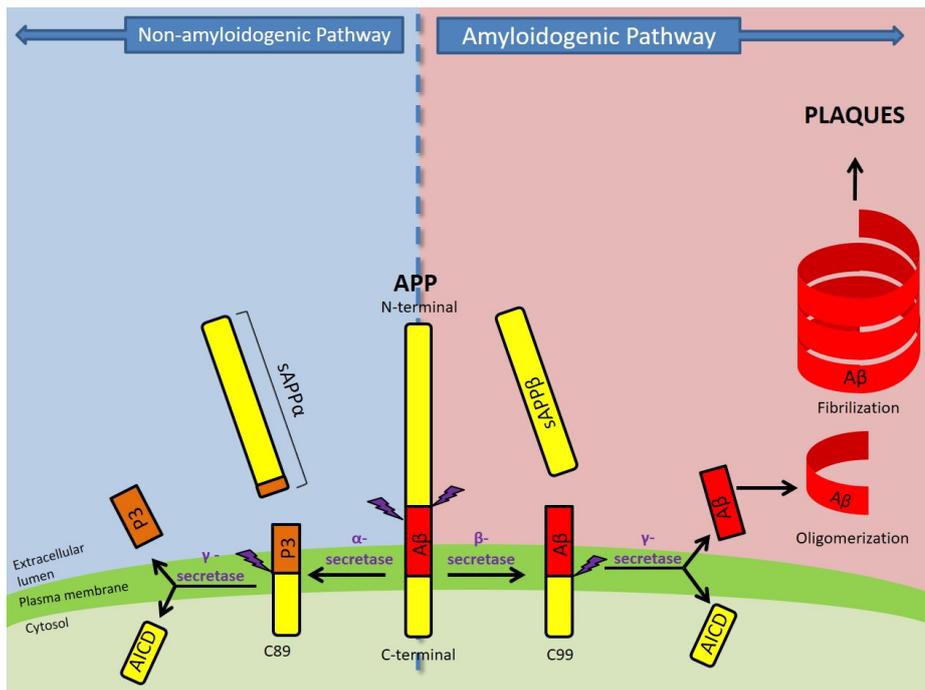
Figure 1. Amyloid precursor protein (APP) proteolysis. The left portion portrays the non-amyloidogenic pathway whereby APP is first cleaved by α-secretase followed by γ-secretase to form non-toxic P3 fragment and AICD protein. One the right, the amyloidogenic pathway depicted involves cleavage of APP via β-secretase followed by γ-secretase to generate functional Aβ peptides. Accumulation of Aβ peptides undergo oligomerisation, fibrilise and eventually form insoluble plaques.

It should be noted that the three main genes posing risk elements for EOFAD are part of the amyloid pathology, namely APP, Presenilin 1 and Presenilin 2 (14).

Neurofibrils and tauopathy

The second hallmark of AD is the presence of neurofibrillary tangles (NFTs). These tangles originate from hyperphosphorylation of the protein Tau. Tau protein can be found in other neurodegenerative diseases such as Parkinson's disease and Huntington's disease. Due to this, AD is considered to be a tauopathy (15). Similar to the amyloid cascade hypothesis, the exact process of tangle formation and its toxicity towards AD remains elusive. In fact, there is still much debate on the source of Tau neurotoxicity: is it caused by i) the deprivation of functional Tau or ii) the aggregation of NFTs; could iii) soluble Tau oligomers be the culprits, or is iv) Tau isoform expression driving the process? (15). There is also the possibility that it is caused by a combination of these.

Tau protein is a highly soluble microtubule-associated protein (MAP) found generally in neurons as well as in astrocytes and oligodendrocytes albeit at low levels. Its notable roles include stabilising microtubules and axonal transport regulation. As such, Tau aids in neuron structuring besides allowing essential nutrients and proteins to move along the cell. The human Tau gene is located on chromosome 17, about 100kb with 16 exons. In contrast with the extracellular Aβ peptides, Tau proteins are formed within neurons (16).

Tau is phosphorylated at a nanomolar degree to bind onto microtubules of distal axons for microtubule stabilisation and early development of polarity. Microtubules act as trails throughout the neuron, linking the cell body to dendrites and eventually to synapses. In AD patients, Tau experiences conformational changes and misfoldings in addition to hyperphosphorylation that stimulate abnormal aggregation into fibrils in neurons. Instead of being microtubule-bounded, mutant Tau is free-living and ineffective in organising the cytoskeleton. They gather at the somatodendritic compartment as filaments. Fibril formation spreads in a hierarchical fashion to other parts of the brain, starting from the entohinal cortex before continuing to the hippocampal formation,

association cortices and finally the primary sensory zone in advanced phases of AD. Not only that, the mutant protein takes on a completely reversed role by hindering microtubule assembly and disrupting microtubule organisation which ultimately leads to neuronal death (17).

Drosophila melanogaster as a Model Organism

Drosophila's history in science predates over 100 years with Thomas Hunt Morgan pioneering the field (18). *Drosophila* have short lifespans and are considered a four-in-one model due to their life history consisting of distinct morphological stages: the embryo, the larva, the pupa and the adult, each catering to different modelling functions (19). Care and housekeeping call for few equipment with low overall cost.

Its simple anatomy and genetic characteristics aid in its function as the ideal disease model. Male *Drosophila* do not undergo meiotic recombination nor do they possess any synaptonemal complex (20, 21). Hence, recombination control is focused only on females. To simplify things, males and females can be efficiently distinguished under the light microscope and a single female produces hundreds of offspring in days. It was from the

fruit fly that we first discovered the idea that chromosomes carry hereditary traits (18). Its four pairs of chromosomes can be readily viewed as giant polytene chromosomes with thicker regions symbolising transcription activity. Moreover, the discovery of balancer genes which serve to stop heterozygous recombination has benefitted *Drosophila* studies (22).

The *Drosophila*'s genome size of approximately 13,500 genes (23) is minute compared to the human genome of 25,000 protein coding genes (24). From the 287 recognised human disease genes, a total of 197 (69%) possess a *Drosophila* homolog (21). Besides, *Drosophila* have less genetic redundancy compared to vertebrate models making gene characterisation less complicated. *Drosophila* brain has a similar but simpler central nervous system compared to mammals. Both systems consist of neurons and supporting glia with the same neurotransmitters, all of which are protected by a blood-brain barrier indicating that the basic principles of the neural network are conserved from invertebrates to vertebrates. Many cellular processes involved in neurodegeneration including oxidative stress are exhibited in *Drosophila*. *Drosophila* also mimics complex age-dependent behaviours such as memory and locomotor ability (25).
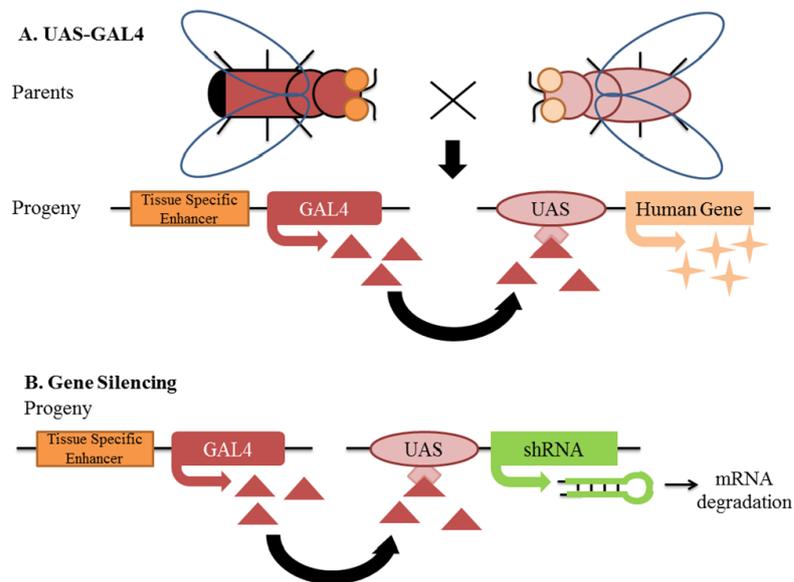

Figure 2. "Humanised" flies expressing transgenic genes using the UAS-GAL4 system. A) GAL4 expressing fly on the left crossed with a UAS transgenic fly fused with a human disease gene. Progenies of the two lines carry both sets of gene sequences. Expression of the human gene is time and tissue controlled reliant on the GAL4 gene sequence. B) Transgenic UAS lines carrying shRNAs interferes with specific gene expression in the presence of GAL4 transcription factor.

To turn *Drosophila* into an AD model, *Drosophila* employed the use of the UAS-GAL4 system (Figure 2). The GAL4 yeast transcription factor is paired with a tissue-specific promoter gene and inserted into a *Drosophila*. On the other hand, the human disease gene is fused downstream from the yeast galactose upstream activator sequence (UAS). This sequence is activated by the presence of GAL4 (26). Commonly, UAS and its partner gene are inserted into a *Drosophila* line different from the GAL4 sequence. Offspring of these lines will produce *Drosophila* that express the human protein in wanted tissues. Such procedures produced GAL4 driver lines including drivers frequently used in neurotoxicity studies such as the pan-neuronal elav (embryonal lethal, abnormal vision) promoter and the eye-specific GMR (Glass Multimer Reporter) driver.

Establishing Drosophila as a model for neurodegenerative diseases

The most crucial aspect to *Drosophila* is its ability to utilise various genetic manipulations that are unfeasible to be carried out in mammals. Traditional forward genetic screens have pinpointed mutations that cause brain degeneration. *Drosophila* mutants including bubblegum (bgm) (27), swiss cheese (sws) (28) and drop-dead (drd) (29) were successfully isolated by screening for *Drosophila* with shortened lifespan before examining their brain pathology.

Forward genetic approach identifies potential genes that trigger known phenotypes through mutagenesis. In random mutagenesis, gene properties are altered via methods that are exclusively random to yield mutations. The goal is to generate large amounts of mutants, each with dissimilar random gene defects (21). Random mutagenesis procedures call for a combination of good screening assays and observational skills (30, 31). Regardless, it was this approach that was awarded the Nobel Prize for successfully screening the early development genes of *Drosophila* (31, 32).

In contrast, reverse genetics is where the functional role of a known gene is determined. The disrupted gene is identified through phenotypic scrutiny. Familial neurodegenerative diseases comprise of recessive mutant genes due to loss-of-functions. To mimic this, endogenous *Drosophila* homologs of human disease genes are disrupted via transposon mutagenesis or RNA interference (RNAi). P-elements (*Drosophila* transposons) modify its location by

"jumping" within the host genome (33). When altering its position, transposons duplicate and spread; sometimes even reversing its own mutations (34). P-elements are easily retraceable and provides the user with a straight-forward mapping of the insertion site. (31, 33). RNAi silences the selected gene and transcription of the protein is effectively cancelled. The UAS-GAL4 system has enabled researchers to tap into gene silencing by combining the system with RNAi technology. Instead of a human gene, a short hairpin RNA (shRNA) sequence compliments the UAS promoter (Figure 2). Once shRNAs are transcribed, they are processed into short interfering RNAs (siRNA). Further processing and aid from other proteins allow the siRNA to recognise and degrade the target mRNA (35, 36). With respect to neurodegenerative studies, this paired system has allowed for the investigation of side effects caused by gene inactivation towards post-mitotic cells in the *Drosophila* nervous system (31). As opposed to usage of the UAS-GAL4 system alone, gene silencing "loses" the specific phenotypes when mRNA is removed.

On the contrary, modifier screens benefit from both forward and reverse genetics. These screens are conducted to identify proteins or genes in addition to pathways that control disease pathologies. Almost all loss-of-function mutations are recessive. Nonetheless, once a particular pathway has been disrupted by another mutation before the addition of the loss-of-function mutation, the genes associated with the process may be modified—either being enhanced or suppressed. To prepare the screen, a *Drosophila* line carrying a genotype expressing a "borderline" phenotype is required that is easily observable and sensitive to genetic modifications. The line is then crossed with another *Drosophila* line containing the loss-of-function mutation (21, 37, 38). In neurodegenerative investigations, neurotoxicity is commonly observed through the rough eye phenotype (REP). The degree of severity of REP from wild type eyes indicates the neurotoxic potency of the disease. After crossing with a loss-of-function mutation *Drosophila* line, offspring are then observed for phenotypic divergence from the REP appearance (31).

For example, the *Drosophila* Tau model has an advantage over other animal models as targeted expression of Tau proteins in adult *Drosophila* retina leads to modifications in the external eye structures, among them are reduction of external eye size, loss of ommatidia organisation and defects of the interommatidial

bristles. The REP correlates with retina cell loss such as photoreceptors. It aids observation on Tau neurotoxicity as expression levels of *Drosophila* Tau (dTau) is high in the retina (15). Additionally, Tau REP is not essential for *Drosophila* survival and is not only highly sensitive but can be easily recorded when assessing effects of phenotype owing to genetic manipulations (39).

Considerations and challenges in using Drosophila

Although *Drosophila* is an excellent instrument in studying the underlying processes such as core physiological and cellular processes, it is lacking as a model for more complex processes. Another prominent setback is the different anatomical structure between *Drosophila* and humans. For instance, both organisms have contrasting blood-brain permeability and *Drosophila* only has haemocytes as blood cells. Also, genome comparison shows that humans have a greater microsatellite mutation degree while *Drosophila*'s nucleotide diversity is at a higher peak. There is also a risk when using *Drosophila* to model diseases whereby pathogenic causes may be vertebrate-specific which do not translate well in to the invertebrate *Drosophila* (37).

Turning *Drosophila* into an AD model

Drosophila has aided AD studies in uncovering crucial mechanisms and pathways. A summary of the numerous *Drosophila* models established for both amyloidosis studies and tauopathy in found in Table 2. Most of the genes have a *Drosophila* homolog with identity between 28%–53%. However, what is important is that *Drosophila* recapitulate the phenotype seen in the AD patients.

Drosophila's contribution to understanding AD amyloidosis pathogenesis

Most AD-linked genes have a *Drosophila* counterpart. As such, *Drosophila* carries a human APP counterpart known as dAPPl (*Drosophila* APP-like). When dAPPl expression was silenced, *Drosophila* exhibited memory loss reminiscence of AD symptoms. Expression of human APP in the same *Drosophila* managed to revert the phenotype indicating that the conserved region of APP is vital for long-term memory (48, 49). Inversely, overexpression of dAPPl resulted in the impairment of axonal transport (50). Similar to the human non-amyloidogenic pathway, dAPPl can be cleaved by the alpha-secretase homolog, Kuzbanian (Kuz) (51). Also, gamma-secretase complex components are conserved in the *Drosophila* with roles in Notch signaling (44, 52).

The Aβ region is not conserved from humans to *Drosophila*. Thus, there is no production of the gene in wild type *Drosophila* (14). In addition, *Drosophila* do not possess the human beta-secretase (BACE) enzyme. Instead, a beta-secretase-like enzyme (dBACE) was recognised to have 25% similarity to human BACE1 enzyme and 28% identity to human BACE2 (A more concise comparison of human genes involved in amyloidosis pathway and their *Drosophila* homologs can be found in Table 3 and Figure 3). Despite this, *Drosophila* was able to recapitulate many aspects of AD. Overexpression of dBACE enzyme cleaves human APP to produce senile plaques (45, 53). Fascinatingly, dBACE overexpression splices dAPPl protein at a site corresponding to the Aβ peptide. This fragment aggregates in an age-dependent manner besides inducing behavioural changes and neurodegeneration. This suggests that the amyloidogenic pathway is well maintained (14, 51, 54).

Table 2. Summary of AD *Drosophila* models including their phenotypes

Related genes	Fly homolog	Phenotypes/ *Drosophila* model	References
Aβ protein	None	Eye degeneration, amyloid plaque formation, vacuolation of brain, reduced lifespan, locomotor defects.	(39–44)
Presenilin 1 and 2	dPsn	Pupal lethality, dorsoscutellar bristle duplications, wing notching and wing vein defects.	(45)
Tau	dtau	Eye degeneration, disruption of microtubule network, axonal degeneration, morphological changes in neuromuscular junctions.	(40, 46)

Table 3. Genes involved in human APP proteolysis and their functions in addition to their *Drosophila* homolog with their accession number and percentage identity. The *Drosophila* homologs are blasted against their human genes at http://www.ncbi.nlm.nih.gov/on 15/8/2016

| Human gene	Accession number	Function	*Drosophila* homolog	Accession number	Identity (%)
Amyloid Precursor Protein (APP)	P05067.3	An integral membrane protein containing the Aβ region. Its *Drosophila* homolog lacks this region. The principal function APP remains unknown.	dAPPl	AAD55414.1	36%
ADAM 10	O14672.1	A member of the ADAM cell surface proteins. It is the most crucial enzyme in neurons to possess α-secretase activity. It cleaves APP within the Aβ region.	Kuzbanian	P07201.3	28%
BACE1	P56817.2	An aspartate protease with a role in the myelin sheath formation in neuronal cells. It cleaves APP at β-secretase sites of Asp^{+1} to initiate Aβ production.	dBACE	NP_609253.1	26%
BACE2	Q9Y5Z0.1	A close homolog of BACE1. It is believed to be a protease functioning in pathways leading to Alzheimer's disease.	dBACE	NP_609253.1	29%
APH1	Q96B13.1	Modulates the maturation of Presenilin and is vital in the assembly of the γ secretase complex.	dAPH 1	AAF51212.1	47%
Presenilin 1	P49768.1	Encompasses the catalytic core of the γ secretase enzyme.	dPresenilin 1	AAF56349.2	53%
Presenilin 2	AAP35630.1	The final component to complete the γ secretase assembly. Its participation induces a conformational change in the complex that leads to the maturation of γ secretase as an active enzyme.	dPresenilin 2	A86BE9.3	53%
Nicastrin	NP_056146.1	Promotes maturation and appropriate trafficking of the units in γ secretase. Regulates neprilysin in its role in degrading Aβ protein.	dNicastrin	AAF56349.2	31%
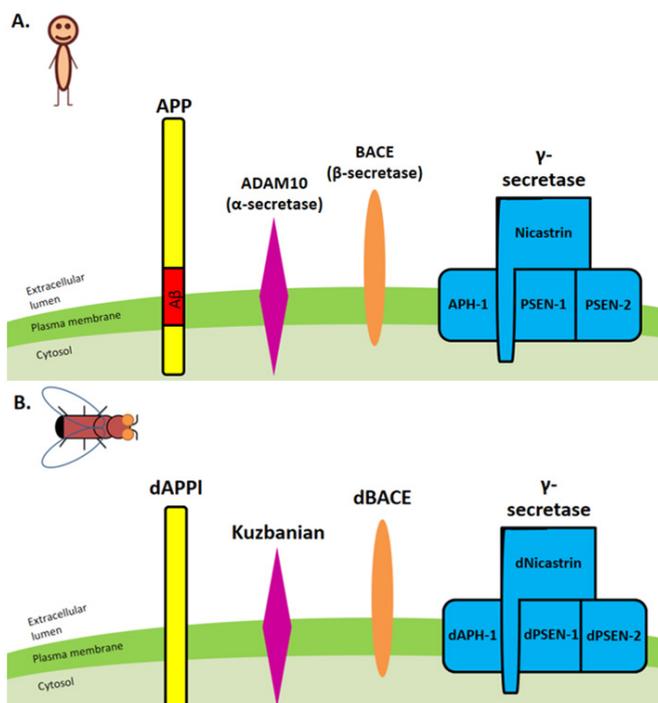
Figure 3. A schematic comparison of the components involved in APP proteolysis between A. humans B. *Drosophila*.

Since *Drosophila* does not endogenously generate Aβ peptides, the first AD *Drosophila* model employed the UAS-GAL4 system to produce triple transgenic *Drosophila* expressing the human APP, BACE and *Drosophila* presenilin (dPsn) with point mutations that parallel EOFAD mutations N141I, L235P and E280A (37, 45, 51, 55). *Drosophila* demonstrated age-dependent neurodegenerative phenotypes including photoreceptor cell loss and decreased longevity. The co-expression of human APP and human BACE caused the formation of Aβ40 and Aβ42 plaques that precedes the onset of neurodegeneration while co-expression of dPsn mutants hastens photoreceptor degeneration (37).

In a more straightforward approach for Aβ-toxicity studies, either Aβ40 or Aβ42 peptides were attached to the N-terminal of the signal peptide of a *Drosophila* endogenous necrotic gene sequence. This ensures secretion of intact Aβ species in the *Drosophila* brain. Complimented by the UAS-GAL4 system, expression of the Aβ fragments is spatiotemporal. The expression is also parallel to signal peptide levels generated by amyloidogenic processing of APP, therefore erasing any factors from APP processing. Concisely, this system provides a pioneering method to directly assess the toxicity of either Aβ42 or Aβ40 separately (14, 51, 55).

Aβ40 and Aβ42 are the most common Aβ isoforms that aggregate in *Drosophila* brain. However, only Aβ42 produce deposits which cause neuronal dysfunction and severe neurodegeneration in absence of truncated amyloid plaques or formation of neurofibrillary tangles. Contrariwise, Aβ40 *Drosophila* had learning dysfunctions without amyloid deposits, indicating that prefibrillar oligomers or soluble protofibrils might be the culprits behind cell death (56).

There is a temporal relationship between the aggregations of intracellular Aβ42 and extracellular non-amyloid plaques. Expression of Aβ42 with the Arctic mutation (E22G) which enhances Aβ protofibril formation and intracellular Aβ accumulation showed that the onset of neurodegeneration is inversely proportional to Aβ42 oligomerisation. Also, locomotor dysfunction affiliated with the build-up of intracellular Aβ42 was observed prior to plaque generation. Despite this, Congo Red which blocks Aβ oligomerisation was able to rescue the shortened lifespan. Again, the data showed that intracellular Aβ and extracellular

soluble Aβ cause neurodegeneration (14). This debunking of the common consensus whereby Aβ40 and Aβ42 peptides are the root behind toxicity in AD pathogenesis has also been shown in previous studies utilising other animal models (57, 58).

A particularly recent *Drosophila* model involves the apolipoprotein E (ApoE) gene. In humans, the immunoreactivity of ApoE protein has been correlated with amyloid plaques. Humans carry two copies of this gene with various combinations of the three ApoE alleles: ε2, ε3 and ε4. The ε4 allele poses the greatest genetic risk factor for sporadic late-onset AD while ε3 is the most frequent and neutral isoform. In *Drosophila,* there is currently no known orthologue for this gene (59). With the use of the UAS-GAL4 system, human APoE alleles ε3 and ε4 can be overexpressed in the *Drosophila* brain. The model exhibits progressive neurodegeneration, memory impairment and early death. In the central nervous system (CNS), neuronal injuries lead to a surge in human ApoE proteins levels suggesting a role for ApoE in repairing the nervous system (60).

Presinilin genes are found to be pathogenic loci linked to EOFAD. They are catalytic subunits of gamma-secretase found to be associated in mitochondria-endoplasmic reticulum coupling (61). Alteration in gamma-secretase cleavage changes production of total Aβ fragments formed (13). *Drosophila* has demonstrated that presinilin is vital for ligand-dependent nuclear access intracellularly (52). Furthermore, the model carrying loss-of-function mutations of *Drosophila* Presinilin gene led to phenotypes similar to the lethal Notch gene which verifies the need of presinilin for normal proteolytic production of Notch fragments (44).

Drosophila's presence in Tau toxicity studies

Drosophila possess a single endogenous gene (dTau) homologous to a microtubule binding domain (MTBD). dTau is 46% identical and 66% similar to its human counterpart and is the sole MAP1A associated protein in *Drosophila*. However, it lacks N-terminal functional domains found in mammalian MAPs. *Drosophila* Tau locus is mapped onto the location 98A6 on the third chromosome's right arm and is encoded by 7 exons spanning about 16kb (62).

dTau is an indispensable gene for *Drosophila*. Knockdown of dTau in neurons resulted in premature death with only 3% of

mutant *Drosophila* surviving till adulthood. This was partially rescued by the expression of human Tau, hence there is a functional conservation of Tau from *Drosophila* to humans. Deprivation of dTau in neurons has led to progressive degeneration particularly in photoreceptors (15). dTau is required for late stages of photoreceptor development as the same deterioration phenotype was observed when knockdown of dTau was limited to adult *Drosophila*. The gene was also found to be necessary for microtubule stabilisation and rhabdomeric membrane extension in addition to microvilli formation. Overexpression of wild type vertebrate Tau or dTau in adult mushroom bodies resulted in defective olfactory learning and memory but interestingly no neurodegeneration phenotypes were observed (63). Subsequently, behavioural plasticity deterioration signifies the earliest neuropathological symptoms of tauopathy (64).

Overexpression of the human Tau, be it mutant or wild-type, in *Drosophila* was able to mirror symptoms seen in Tau-related AD patients: age-dependent progressive neurodegeneration characterised by nuclear fragmentation and vacuolisation in the brain complemented with the trademark phosphorylation of Tau which ultimately caused premature death. Despite this, there was no large aggregation of filaments found in *Drosophila* (51).

Glycogen synthase kinase 3β (GSK-3β), a member of the Wnt signalling pathway, and other components of the pathway have been associated with AD-linked proteins. GSK-3β overexpressing *Drosophila* exhibited heightened neurodegeneration, increased pathogenic phosphorylation of Tau in addition to the formation of intracellular inclusions that are comparable to NFTs that were absent in previous Tau *Drosophila* models. This correlates with prior knowledge whereby Tau hyperphosphorylation accelerates aggregation (47).

Through loss-of-function and overexpression approaches, the partitioning defective-1 (PAR-1) protein was linked to taoupathy as a Tau kinase that regulates Tau phosphorylation (65). The mammalian counterparts of PAR-1, MARK proteins (66) also function in cellular and developmental processes while being bound to neurofibrils in AD brain. MARK overexpression in *Drosophila* resulted in increased Tau phosphorylation at specific sites of Ser262 and Ser356, therefore augmenting Tau toxicity. In contrast, knock-out of PAR-1 or

mutants of PAR-1 phosphorylation sites lead to the termination of Tau toxicity. Furthermore, PAR-1 was found to initiate a phosphorylation process via multiple sites to generate toxic Tau. PAR-1 phosphorylation at the same Ser262/Ser356 sites is mandatory for downstream phosphorylation (65).

Besides being a MAP, Tau is connected to another part of the cytoskeleton, the filamentous actin (F-actin), and acts as actin crosslinking proteins. Hirano bodies are paracrystalline actin-rich inclusions and are found in most neurodegenerative patients' brains including AD (67). They age-dependently increase in concentrations, akin to Tau proteins. *Drosophila* models proved that Tau toxicity is linked to F-actin and Hirano bodies. Modifying F-actin levels resulted in extreme alterations of Tau-induced neurodegeneration (68).

When testing the relationship between NFTs and Aβ, *Drosophila* expressing phosphorylated human Tau and Aβ42 demonstrated disrupted transport of axonal cargo and dysfunctional neuroendocrine wherein they were unsuccessful in expending their wings as well as hardening their cuticles. This *Drosophila* had heightened Tau phosphorylation and magnified Tau-mediated phenotypes including premature death and behavioural defects. Treatment with GSK-3β inhibitor, LiCl, rescued these phenotypes; suggesting that GSK-3β functions in the co-expression of Tau and Aβ42 to cause neuronal dysfunction (69). Intriguingly, Ser262 needed for Tau phosphorylation is activated by the DNA damage-activated Checkpoint kinase 2 (Chk2) while stimulation of Chk2 protects against Aβ42 toxicity. Thus, DNA repair pathway stimulation prevents Aβ42 toxicity but initiates Tau phosphorylation (70).

The identification of PAR-1 in its link with tauopathy has been crucial in revealing signaling mechanisms. *Drosophila* models have shown that phosphorylation of PAR-1 by the tumour-suppressor protein LKB1 (also known as serine-threonine kinase 11, STK11) is necessary for PAR-1 activation. The role of LKB1 was formerly unknown. Through this study, it was revealed that various stress stimuli including the overexpression of human APP can promote PAR-1 activation and thus Tau phosphorylation in an LKB1-dependent manner. In addition, Tau hyperphosphorylation have been seen in human AD brain (71) and also in APP-Tau transgenic mouse models (72). These further evidence that LKB1/PAR-1/Tau phosphorylation could be the

missing puzzle between amyloid plaques and Tau lesions in AD pathogenesis (64). Figure 4 summarises the proposed mechanisms behind AD toxicity that links APP and Tau proteins.

Therapeutic Drug Discovery

The main objective in establishing animal models for human diseases is to provide new insights into disease pathology that can be directed to finding cures of said diseases. Here we discuss some of the therapies discovered from *Drosophila*. Many *Drosophila* models were developed for various drug testing purposes. One such model tested on memory assessment in AD studies. Epidermal growth factor receptor (EGFR) signalling at a certain range is required to sustain homeostasis environment in *Drosophila* mushroom bodies mandatory for brain plasticity, learning and memory (73). Over-activation of EGFR leads to increased Aβ-induced short-term memory loss. The same *Drosophila* model proved that EGFR inhibitors, geftinib and erlotinib were unsuccessful in preventing memory loss in Aβ42 *Drosophila* but had positive results with memantine, a dementia treating drug (74).

Incidentally, *Drosophila* is also an ideal model to test natural products for AD treatment. For instance, first *Drosophila* model to associate *Ginkgo biloba* with AD verified that the plant has an effect on mitochondrial dysfunction that comes with the Aβ cascade. *Ginkgo biloba* improves metabolic energy pathways through enhancement of the mitochondrial

coupling state. Furthermore, the model showed substantial results for *Ginkgo biloba* as a long term therapeutic drug for AD (75).

An increase of Aβ42 above a threshold level in the brain is considered the main event in AD pathogenesis. Thus, steps to develop disease-modifying therapies focused on reducing Aβ42. In regards to the amyloidogenic pathway, targeting relevant secretases is a feasible option. Both β-secretase and γ-secretase inhibitors ameliorate Aβ toxicity in triple transgenic *Drosophila* expressing the human APP, BACE and dPsn (37, 45, 51, 55). In another *Drosophila* model, curcuminoid compounds from *C. longa* rhizome was able to rescue the REP induced in *Drosophila* expressing APP and BACE-1 (76). Thus, revealing the potential for *C. longa* rhizome-derived compounds as therapeutic drugs for AD.

AD *Drosophila* models were also used to study microRNAs (miRNAs) that post-transcriptionally regulate gene expression and silences RNA. Numerous miRNAs in the brain of AD patients were discovered to be impaired; for instance, miR-124 that is vital for neurogenesis and neuropathology were downregulated (77). Surprisingly, there is only one locus for miR-124 in *Drosophila* compared to its three loci in vertebrates which eases miR-124 knockout in *Drosophila* (78). AD *Drosophila* had similar downregulated miR-124. The over-expression of miR-124 was able to rescue locomotive degeneration in AD *Drosophila*. Also, miR-124 was found to have an effect on Notch signalling pathway. miR-124 knockout *Drosophila* had
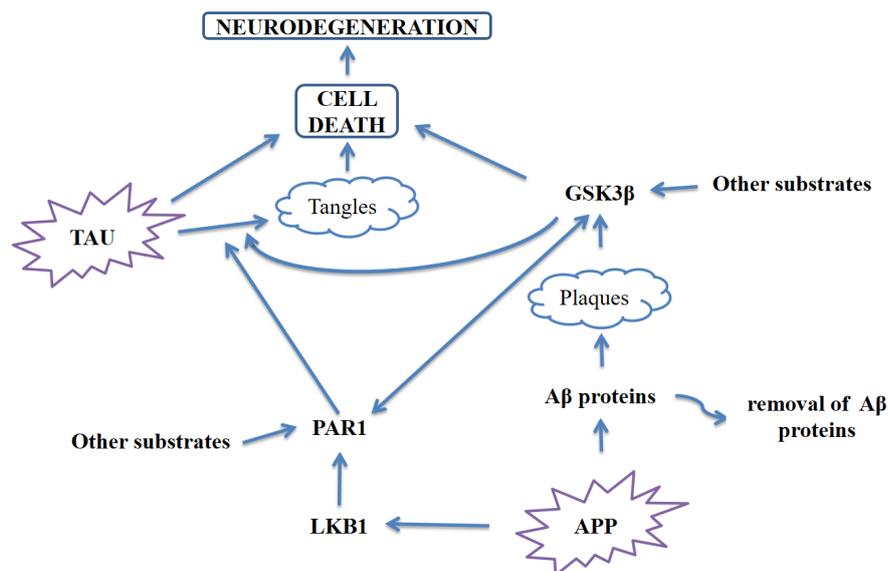

Figure 4. Proposed pathogenesis of Alzheimer's disease.

elevated levels of Presenilin and Notch signalling pathway members. Addition of a Notch pathway inhibitor rescued the shortened lifespan of AD *Drosophila* (78). Thus, Aβ expression can be regulated by targeting miR-124.

Another microRNA, miR-219, functions in Tau pathology. miR-219 is involved in neuron differentiation and axon development (79). In the Tau *Drosophila* model, miR-219 uniquely interacted with the Tau 3'-UTR. There is an inverse correlation between Tau protein synthesis and miR-219 levels as nerve growth factor (NGF) temporarily downregulates miR-219 which returned to basal levels once cells fully differentiate. Conversely, lentiviral expression of miR-219 reduced levels of Tau protein and mRNA (80). Both results suggest that miR-219 regulates NGF-induced Tau synthesis and manipulating miR-219 levels could act as Tau treatment.

Conclusion

Overall, transgenic *Drosophila* models have successfully provided valuable information on AD. *Drosophila*'s ability to utilise various genetic tools has allowed in-depth studies on familial aspects of both diseases. Furthermore, due to its simple brain anatomy, *Drosophila* is ideal for *in vivo* testing and screening of therapeutic compounds. We anticipate that, as techniques and analysis progress, *Drosophila* models will assist in the development of disease treatment and ultimately a cure for both diseases.

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Authors' Contributions

Conception and design: FTHP, GMA
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