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Insights from *Drosophila melanogaster* model of Alzheimer's disease

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1. ABSTRACT

Alzheimer's disease (AD) is a common chronic neurodegenerative disease that mainly affects the medial temporal lobe and associated neocortical structures. The disease process involves two abnormal structures, plaques and tangles, which damage and destroy nerve cells. Tangles are twisted fibers of tau protein that build up inside cells. Plaques are deposits of a protein fragment called amyloid-beta (Aβ) that accumulate in the spaces between nerve cells. Aβ derives from the amyloid precursor protein and is the main component of amyloid plaques in the AD brain. Although AD has been extensively examined, its pathogenetic mechanisms remain unclear and there are currently no effective drugs for this disorder. Many AD model systems have recently been established using *Drosophila melanogaster* by expressing the proteins involved in AD in the brain. These systems successfully reflect some of the symptoms associated with AD such as the onset of learning defects, age-dependent short-term memory impairment, increase of wakefulness and consolidated sleep disruption by expressing human Aβ42 or human *APP/BACE* in *Drosophila* central nervous system. We herein discuss these *Drosophila* AD models.

2. INTRODUCTION

Dementia comprises a set of symptoms including poor memory and difficulty with learning, and according to the WHO, Dec 2017, approximately 50 million individuals have dementia worldwide, with nearly 10 million new cases being reported each year. Alzheimer's disease (AD) is responsible for approximately 50-70% of all cases of dementia, with a new case of AD occurring globally every 66 seconds. New cases of AD are predicted to occur every 33 seconds by 2050, resulting in nearly 1 million new cases each year (1). However, effective strategies to control progression and prevent AD have yet to be developed. The selection of models that are advantageous for AD research will provide novel directions and better results. Although various higher vertebrate models have been in use to study the pathophysiology of this disease. However, these models show some limitations such as ethical

restrictions, high cost and length of time associated with generating their transgenic forms, difficulty in maintenance of large quantity of progenies and lesser reproducibility (2). Thus, complementary model organisms have been exploited to obtain more insight into the disease mechanisms. *Drosophila* are small in size and its brain with a simple and well-organized anatomical structure is similar to the human brain in basic aspects of cell biology, gene expression regulation, neuronal connection, cellular signaling, synthesis and cell death (3). Importantly, with the *Drosophila* model we can study sleep/wake behavior unlike mice which have comparatively more fragmented sleep. Furthermore, we can perform learning, memory assays and movement coordination tests and high throughput screening of the therapeutic potential for treatments with this model (4). In this review, we describe the usefulness and limitations of the *Drosophila* system as a model for the study of AD. Biological similarities between humans and *Drosophila* have been exploited with great success in the research field of neurodegenerative diseases, particularly AD (5).

3. ALZHEIMER'S DISEASE (AD)

AD was initially described in a case report by Dr. Alois Alzheimer in 1906. The patient showed memory loss, paranoia, and psychological changes, while shrinkage in and around nerve cells in the brain was noted in an autopsy. The characteristic features of AD are the appearance of extracellular amyloid-beta (Aβ) plaques and intracellular neurofibrillary tangles, neuronal death, and the loss of synapses, all of which contribute to progressive cognitive decline (6). Many hypotheses have been proposed for AD, including Aβ, tau, cholinergic neuron damage, inflammation, and oxidative stress (7).

Hypotheses for Aβ are based on the amyloid precursor protein (APP) and the abnormal cleavage of its peptides, which is the main reason for the formation of Aβ. Abnormal cleavage increases the production of the less soluble and more toxic Aβ42 over Aβ40 (8). Presenilin is the catalytic subunit of γ-secretase, which cleaves and releases the Aβ peptide from the C terminus of APP. Mutations in the *presenilin-1* (*PSEN1*) and *presenilin-2* (*PSEN2*) genes may cause dominant familial AD (9). Mutation clusters have been detected around the γ-secretase cleavage site, and the most famous APP mutation causes a change in amino acids adjacent to the BACE1 cleavage site (8).

A previous study reported that dominant mutations in the *MAPT* gene encoding tau are associated with familial frontotemporal dementia, which supports the abnormal tau protein being directly involved in the pathogenesis of AD (10). Moreover, the abnormal phosphorylation of tau may play a role in the formation of abnormal neurofibrillary structures. Tau undergoes various post-translational modifications, including phosphorylation, arginine monomethylation, lysine acetylation, lysine mono- and dimethylation, and lysine and serine ubiquitylation (7). Furthermore, an extensive genetic study on AD revealed that at least 20 genes play a role in the development of the disease, which is more than that previously predicted (11).

4. DROSOPHILA AD MODELS

Research on AD employs animal models to gain insights into the pathogenetic processes occurring in the AD brain. The genetic screening of *Drosophila* models of AD has been focused on understanding the biological pathways by which dysregulated Aβ and tau production and aggregation likely induce neuronal dysfunction and death. As mentioned above, the *Drosophila* model has some advantages compared to the vertebrate model, but it also has some disadvantages. For instance, we cannot test hippocampal-dependent cognitive functions with this model which are impaired early in human AD. The lack of some homologous structures of human brain is a major limitation of this model (12).

4.1. Aβ model

Among the alterations occurring in the AD brain, including atrophy, amyloid plaques, and neurofibrillary tangles, Aβ has remained the most likely pathway causing AD. The causality of AD is widely accepted to be due to the toxicity of plaques that have accumulated. After being generated, Aβ may contribute to the pathogenesis of AD through several mechanisms: structure-related toxicity, interactions with the immune system, or the

conventional pathway of neurofibrillary tangles (13). The effects of Aβ depend on its size, which has been categorized into oligomers, diffusible ligands, and protofibrils based on its length (14). Protofibrils, the premature amyloid, induces toxicity (15). In addition, this newly formed structure induces the production of nitric oxide and calcium penetration, which lead to cell death (16). Aβ oligomers, particularly the soluble forms of them, are toxic. The oligomers adversely affect long-term memory and learning (17) by producing new proteins and promoting the reformation of dendritic cells, which have been demonstrated in animal models (18). Therefore, the various forms of Aβ contribute to the pathogenesis of AD in different manners.

Besides the main protein Aβ, some models, particularly those using *Drosophila melanogaster*, have demonstrated the protective role of APP. The *Drosophila* model has been widely used as a tool for studying genetics and developmental biology, searching for novel biomarkers for human diseases by genetic screening, and the high throughput screening of drugs for human diseases. *D. melanogaster* has a relatively rapid life cycle of ten days; 1,000 individuals may be simultaneously used, thereby allowing large-scale genetic and physical screening, which may also contribute to the analysis of complex multigenic disorders in the near future (19). Furthermore, 77% of human disease genes in the Online Mendelian Inheritance in Man database match *Drosophila* sequences (20). Therefore, *D. melanogaster* is emerging as one of the most effective tools for analyzing the functions of human disease genes, including those responsible for developmental and neurological disorders (19). Transgenic flies have been produced for the study human Aβ peptide-induced amyloid formation and neurodegeneration by several approaches using the GAL4/UAS system (fig1).

Transgenic flies expressing human Aβ42 in the nervous system exhibited age-dependent short-term memory impairment and neurodegeneration (3). A subsequent study revealed that a mutation in the *Drosophila neprilysin* gene suppressed Aβ42-induced phenotypes by decreasing Aβ42 peptide levels, thereby supporting the role for neprilysin in the catabolism of Aβ peptides *in vivo*. The *Drosophila* model may also be suitable for investigating Aβ metabolism and toxicity at the genetic level (21). The *Drosophila* APP orthologue, APP-like (APPL), is required for long-term memory (22). APPL may protect *Drosophila* from progressive neurodegeneration (23). Consequently, attention has shifted from pathophysiological Aβ to its precursors and associated secretases. Due to the discovery of the functions of these enzymes, the pathology of AD has been elucidated in more detail (24). By using the *Drosophila* model, Lin *et al.* revealed that the intraneuronal accumulation of Aβ42 induced the age-dependent slowing of neuronal transmission along pathways involving multiple synapses. Thus, besides impairments in synaptic transmission and synaptic plasticity as well as an imbalance in excitation and inhibition in the neuronal network, the slowing of neurotransmission may also contribute to the cognitive deficits associated with AD (25). Although the relationship between Aβ and APP is gradually being clarified due to the discovery of the cutting enzymes described above, the consequence of this connection remains unclear. With the failure of the latest trials, some researchers have changed their hypothesis to amyloid being a consequence rather than the cause of AD (26).

4.2. APP/BACE1 model

This model is ideal for examining modulators of β-site APP-cleaving enzyme 1 (BACE1) or APP metabolism by establishing transgenic flies that carry GAL4-driven constructs encoding human APP and human BACE1. BACE1 has been identified as the secretase that cleaves APP at the N-terminal Aβ site, which is the first step in generating Aβ via the amyloidogenic pathway (fig2). Therefore, BACE1 plays a crucial role in amyloid plaque formation (27). BACE1 also has potential as a therapeutic impact target for AD (23). The enzyme in the last secretase step is γ-secretase, which is the most complex secretase that was recently isolated and has a special cutting position inside the plasma membrane. This protein is complex because its molecular structure comprises the components presenilin-1, presenilin-2, nicastrin, Aph-1, Pen-2, and transmembrane proteins. The combination of β- and γ-secretases is essential for the formation of Aβ amyloid plaques (21).

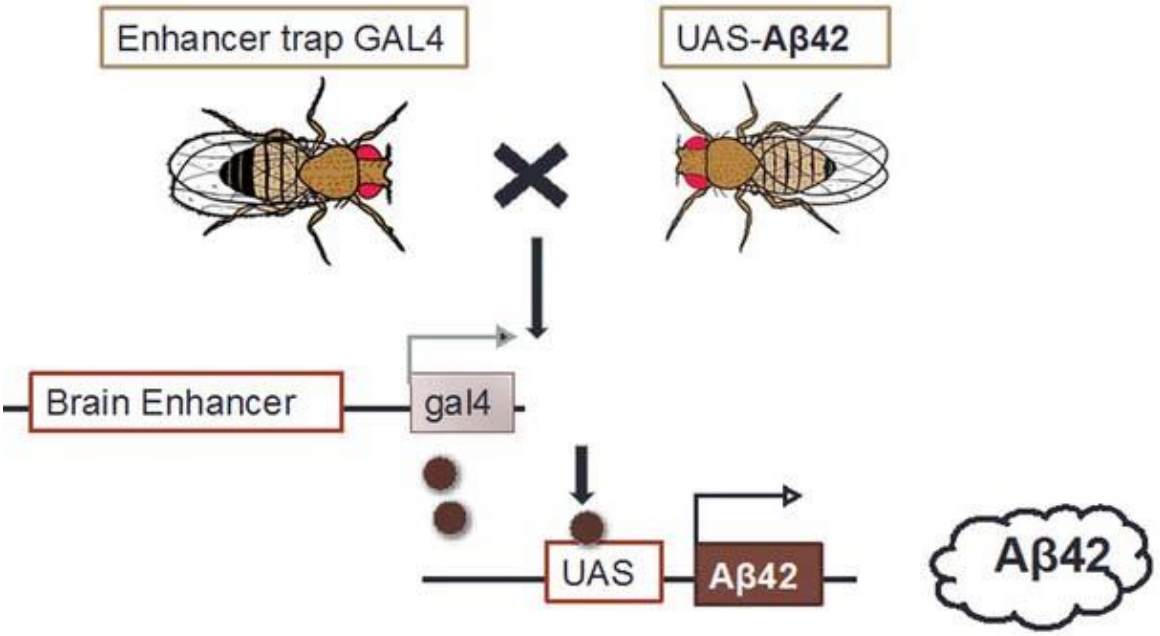

Figure 1. Aβ42 expression in *Drosophila* by the GAL4/UAS system. Driver lines expressing the transcriptional activator GAL4 in a tissue-specific manner are crossed with UAS lines with genomic inserts of a target gene fused to GAL4-binding sites.

In a fly model expressing human APP, the protein is cleaved by transgenic human BACE1 and then by endogenous *Drosophila* γ-secretase to generate Aβ peptides in the brain (28). Aβ peptides processed from human APP have been detected in the retinas of transgenic flies and amyloid plaque formation is mediated the toxic photoreceptor cells (28). A *D. melanogaster* AD model was developed by expressing the human APP695 and BACE genes in the central nervous system (CNS) of *Drosophila* and mimicked a number of AD symptoms including accumulation of Aβ-containing puncta in their brains, decreased dendritic and axonal fields in areas of the brain important for learning and memory, and memory deficits (29). In another study, in which the elav-GAL4 driver was used to express the human APP and BACE transgenes in the pan-neurons of the fly, the *Drosophila* model showed the loss of synaptic and behavioral defects, which is consistent with the findings obtained using mammalian AD models. Larvae expressing human APP and BACE exhibited defects in locomotion that were accompanied by fewer synaptic connections at the neuromuscular junction (NMJ) (30). Furthermore, the expression of human APP and BACE resulted in significant decreases in bouton numbers at the NMJ. A previous study demonstrated that the expression of Aβ42 itself at the larval NMJ also decreased bouton numbers (31). These findings suggest that the simultaneous presence of APP and BACE in the fly model results in the production of high levels of Aβ42. An APP/BACE-expressing fly model revealed the potential of γ-secretase inhibitors as a treatment for AD (30), suggesting that this model is amenable to identifying potential pharmacological agents for the treatment of AD.

The *Drosophila* orthologues of BACE and APP are dBACE and APPL. dBACE is expressed in neurons and axons and is required for glial survival. The loss of dBACE reduces APPL processing and suppresses APPL-induced phenotypes. GMR-GAL4/UAS-APPL and UAS-dBACE expression systems revealed that APPL and dBACE co-localize in the retina (32). Research on APP and BACE-1 has mainly been conducted on non-neuronal cells in vertebrates, and revealed that APP and BACE-1 colocalize in the trans-Golgi network and endosomal

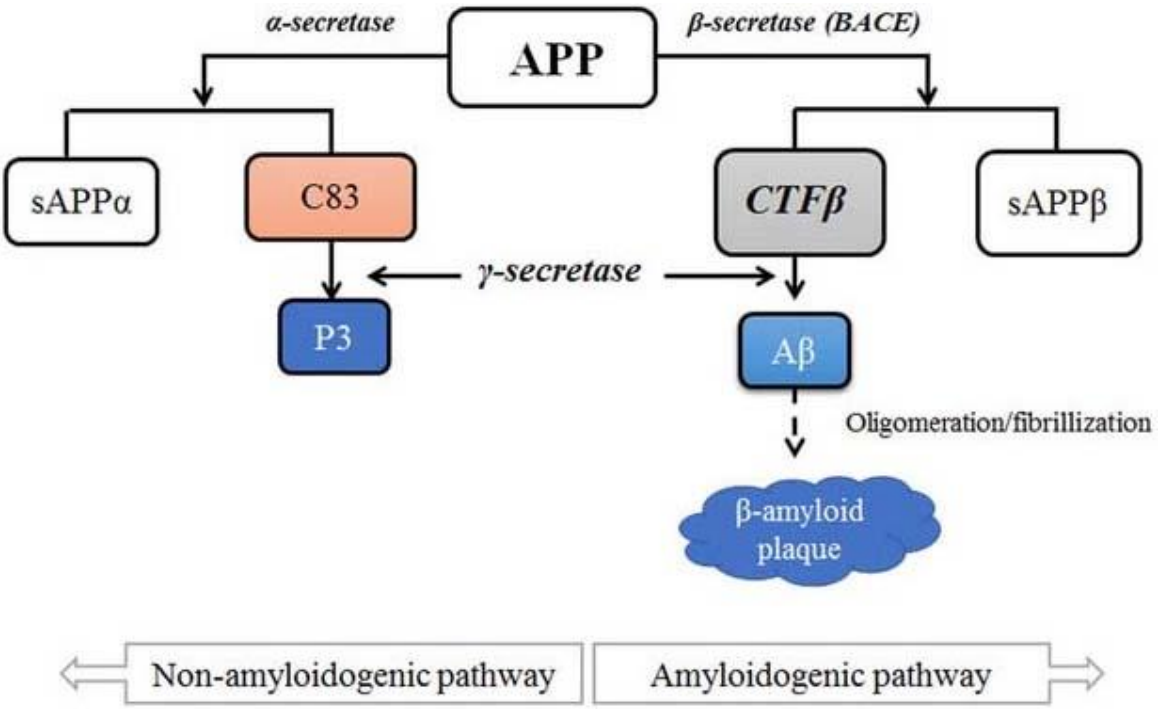

Figure 2. In the amyloidogenic pathway, APP is cleaved by the β-site APP-cleaving enzyme (BACE), a soluble sAPPβ fragment that is secreted outside the cell, leaving a membrane-associated C-terminal fragment - CTFβ (99 or 89 amino acids). CTFβ is then cleaved by γ-secretase, generating the Aβ peptide and a cytoplasmic APP intracellular domain (AICD) (65). The Aβ42 peptide is oligomerized and forms aggregates that accumulate in the brain to form plaques.

compartments (33). On the other hand, mitochondria were mis-localized in the transgenic flies expressing human APP and BACE-1 (34).

There are two BACE variants in vertebrates: BACE1 and BACE2. BACE1 is the principal β-secretase in neurons, while BACE2 cleaves APP more efficiently at sites within the Aβ domain, suggesting that BACE2 limits Aβ secretion (35). In flies, only dBACE cleaves APPL to the predominant β-cleaved C-terminal fragment in order to induce fly Aβ-like fragments, which indicates that fly dBACE and vertebrate BACE share similar functions. The loss of dBACE results in late larval/early pupal lethality in flies (32). In contrast to the fly, BACE1 knockouts in mice are viable (35). This may be due to the presence of the BACE2 protein, suggesting that dBACE plays a dual role for both of the vertebrate proteins. Therefore, APPL/dBACE are useful tools for building an AD research model and may replace vertebrate animal models.

4.3. Tau model

Although Aβ-expressing flies are useful for understanding the molecular mechanism of AD pathogenesis and are frequently used, a model that expresses tau is also very important. Tau is a neuron-specific microtubule-binding protein and a member of the microtubule-associated protein (MAP) family (36). It is required for the integrity and functioning of neuronal cells and acts as a monomer that binds to the microtubule cytoskeleton, thereby stabilizing the microtubule structure to promote its assembly (37). *Drosophila* models of tauopathy have contributed to our understanding of the role of hyperphosphorylation in tau toxicity. Tau localization was demonstrated in motor and sensory neuron axons in *Drosophila* larvae segmental nerves by immunostaining and was shown to be affected by *tau* depletion (38). *Drosophila* has a tau homolog and the pathways involved in tau neurotoxicity such as Wnt, JNK, and TOR are shared with humans. Moreover, tau expression induces learning and memory deficits

in *Drosophila*, mimicking AD in humans (39, 40).

The Gal4-UAS targeted expression system has been used to model tauopathies (41) by overexpressing mutant or wild-type (including different isoforms) human (or *Drosophila*, even bovine or rodent) tau to specific neuronal or glial cells in larvae and adult *Drosophila*. The consequences of tau expression have been investigated by evaluating neuronal functions, such as axonal transport, synaptic function, olfactory learning, and locomotor behavior, and cell loss/toxicity, including eye roughness, lifespan and lethality, brain vacuolization, apoptotic cell death, and the total loss of bristles. Research using these models revealed critical pathogenetic mechanisms by which abnormalities in tau cause neurodegeneration in tauopathies (40, 42–44).

The first studies on *Drosophila* models of tauopathy demonstrated the neurodegenerative effects of tau overexpression in neurons, which recapitulated the pathological phenotypes observed in tauopathies, such as AD (45, 46). A previous study reported that the expression of various tau proteins in mature sensory neurons induced morphological defects, including axon blebbing, axon loss, axon defasciculation, and reduced arborization, which likely represent a degenerative process (45). The expression of wild-type tau proteins exerted a significant effect on life expectancy with an obvious neurodegenerative impact, and this effect was more pronounced with disease-causing mutant tau proteins (46).

The multisite hyperphosphorylation of tau has been implicated in the pathogenesis of neurodegenerative diseases, including AD. Hyperphosphorylation associated with neuronal dysfunction and neurodegeneration has been demonstrated (45–47). A previous study reported that PAR-1 directly phosphorylated tau at Ser262 and Ser356, which are necessary for the activity of downstream kinases, such as GSK-3/sgg and Cdk5, to phosphorylate other sites in order to generate disease-associated phospho-epitopes. The effects of phosphorylation on tau toxicity suggest new potential routes for therapeutic targets (48). A recent study showed that the resistance of tau to phosphorylation by GSK-3/sgg results in markedly stronger toxicity because this mutant tau exhibited stronger affinity for microtubule binding than wild-type tau. These findings suggest that tau phosphorylation at PAR-1/MARK sites (e.g. Ser262 and Ser356) cause neurodegeneration, while tau phosphorylated at GSK-3/sgg sites (e.g. Ser202, Thr205, Thr212, Ser214, Thr231, Ser396, and Ser404) cause dysfunction, possibly via microtubule-binding effects (49). Bakhoum *et al.* revealed that autophagy may play a role in tauopathies using a *Drosophila* model of human tauopathy, and the misexpression of human tau induced the accumulation of autophagic intermediates with large vacuoles, named giant autophagic bodies (GABs), which suggested autophagic dysfunction. Furthermore, the induction of autophagy may rescue the tauopathy phenotype, suggesting that the formation of GABs is a compensatory mechanism rather than a trigger of neurodegeneration and also that the disruption of autophagic processes plays a critical role in the progression of the tau pathology (50). Another key mechanism of tauopathy is protein glycosylation, which appears to be altered in the AD brain (51–53). The role that protein glycosylation plays in AD pathology currently remains unclear. *Drosophila* overexpressing tau in the compound eye was used to examine the relationship between eye phenotype and the expression levels of fly homologs of glycosylation-related genes identified by bioinformatics studies of human genome (54). These experiments identified glycosylation genes that may augment or ameliorate tauopathy phenotypes, suggesting that OstDelta, l(2)not, and beta4GalT7 are tauopathy suppressors, whereas pgnat5 and CG33303 are enhancers of tauopathy. These findings indicate that specific alterations in protein glycosylation play a causal role in the progression of AD, and present potential therapeutic targets (54).

The findings of the first studies to combine human genome-wide association studies (GWASs) with functional validation in *Drosophil*a were recently reported. In these studies, 19 candidate genes out of the 17 Alzheimer-associated genomic regions identified in an autopsy cohort were shown to have conserved orthologs in *Drosophila* (55). Of the 19 genes evaluated in the *Drosophila* model, 6 show interactions with tau toxicity *in vivo*, including

rs10845990 within SLC2A14. SLC2A14, encoding a glucose transporter, is an interesting biological candidate considering the well-known dysregulation of glucose metabolism in the AD brain and likely pathogenetic role of oxidative stress (56).

5. A TOOL FOR SCREENING THERAPEUTIC DRUGS

Personalized medicine aims to treat each patient with the best drug exhibiting optimal therapeutic benefits with minimal side effects. Several advantages of the *Drosophila* model for this purpose are summarized below. These flies breed rapidly, are easily maintained in vials on inexpensive food, and are also cheap to experiment on. It may also be advantageous for testing drug efficacy and toxicity. Therefore, *Drosophila* is a proven and powerful model not only for understanding the basic biological mechanisms of human diseases, but also for drug screening. Since Aβ and Tau were identified as the causes of neuronal changes in AD patients, many studies have focused on the searching for the inhibitor of Aß or Tau. The expression of the human APP and BACE proteins in presynaptic motor neurons at the *Drosophila* NMJ significantly affected the development and morphology of this NMJ. The larvae of this model were treated with the γ-secretase inhibitor L685,458, which rescued these phenotypes (34). These findings suggest the potential of this model for identifying pharmacological agents that may be used in the treatment of AD.

Previous studies evaluated the neuroprotective effects of medicinal plant extracts and natural products against human microtubule-associated protein tau (hMAPT), which induces neurotoxicity in a *Drosophila* AD model. These herbal medicinal products are used as a complementary and alternative treatment that temporarily relieves symptoms, delays, but does not prevent the onset of AD, and slows its progression. The identification of new compounds that prevent the formation or degradation of senile plaques and neurofibrillary tangles will contribute to potential treatment strategies and drug development. These plants need to be tested and their toxicity analyzed. Flies will then be cultured and crossed to obtain two groups: wild-type and AD flies expressing the human protein. The two groups will be fed food supplemented with or without the extract. Some assays will then be performed to test the locomotion ability and viability of adult flies or learning and memory ability of third day larvae. A number of natural products, such as blueberries (*Vaccinium spp*.), and green tea (*Camelia sinensis*), have properties that improve brain activity in different manners, making them a primary choice for investigation as natural treatments for AD. The effectiveness of these plants at improving the climbing ability of genetically modified *D. melanogaster* flies expressing the human Tau isoform as well as Oregon wild-type flies has been demonstrated (57). Furthermore, a recent *in vivo* study showed the ameliorative effects of a *Convolvulus pluricaulis* (Shankhapushpi) plant extract on hMAPτ-induced neurotoxicity in a *Drosophila* AD model (58). This plant extract markedly attenuated hMAPτ-induced locomotor deficits, significantly offset hMAPτ-induced early death, and extended lifespans by reducing τ protein levels. The extract also enhanced the levels of endogenous antioxidant enzymes (catalase and superoxide dismutase) and restored the activity of a neurotransmitter enzyme (acetylcholinesterase) (58). Another novel antioxidant compound that was reported to exert neuroprotective effects and ameliorate neurobehavioral deficits in a transgenic *Drosophila* model of tauopathy expressing hMAPT is 4-HIPA (4-hydroxyisophthalic acid), which was isolated from the aqueous extract of *Decalepis hamiltonii* roots (59). This compound at a concentration of 0.1 mg/ml markedly enhanced olfactory memory performance and restored circadian rhythmicity in AD *Drosophila* locomotive behavior to the normal range (59). Therefore, the common mechanism of action of these compounds that underlines neuroprotection involves enhancements in the efficiency of the cellular antioxidant defense system by increasing antioxidant enzyme activities and attenuating oxidative stress.

Drug screening on traditional medicinal plants for neuroprotective activity against Aβ42 cytotoxicity in *Drosophila* models of AD revealed the therapeutic potential of *Polygonum multiflorum* and *Sorbus commixta* for the treatment of AD. *Polygonum multiflorum* also has been used as a tonic and anti-aging agent in many remedies for

centuries to treat cognitive disorders including AD (60). A recent study using low-dose ionizing radiation showed beneficial effects in human Aβ42-expressing *Drosophila* AD models. Th1ey suppressed AD-like phenotypes, including developmental defects and locomotive dysfunction, but did not alter decreased survival rates or the longevity of Aβ42-expressing flies. These findings suggest that low-dose ionizing radiation exerts hormetic effects on the pathogenesis of Aβ42-associated AD (61).

Inhibiting the immune system is also a candidate AD treatment. Aβ still attracts attention, because microglia, innate immune cells in the central nerve, is activated by Aβ accumulation. Toll-like receptors (TLR) play an important role in regulating the response types of Aβ (62). Also by impacting on NF-kB, Aβ induced the production of cytokines and chemokines (63). These findings suggest that the immune system may be a target of AD treatment. Based on extensive research focusing on Aβ, multiple methods, primarily immunological, have been used to develop medication for AD. Several monoclonal antibodies have been developed and are currently in the clinical trial phase, such as solanezmab, a clearing Aβ antibody aimed at solubilizing Aβ. However, significant data have yet to be obtained (64).

6. ACKNOWLEDGMENT

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