Unraveling Alzheimer's Disease Using *Drosophila*

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Abstract

Alzheimer's disease (AD) is a debilitating neurodegenerative disorder that predominantly affects people aged over 65 years. AD is marked by cognitive deficits and memory problems that worsen with age and ultimately results in death. Pathology of AD includes aggregation of the amyloid beta peptide into extracellular plaques and the presence of hyperphosphorylated tau in intracellular neurofibrillary tangles. Given that many factors are involved in the disease along with the ability to study individual aspects of disease pathology under controlled conditions, several genetically tractable animal models have been developed. Despite years of research, treatments remain limited and many therapies that yield

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promising data in animal models fail to translate it in humans. Here, we discuss the use of a highly versatile *Drosophila melanogaster* (*aka* fruit fly) model to study AD. The genetic machinery is conserved from fly to humans. The *Drosophila* eye has proved to be a genetically tractable model to study neurodegenerative disorders and for genetic and chemical screens. We highlight the utility of modeling AD by expressing human Aβ42 in the developing *Drosophila* retina. This system has been used recently to uncover new factors involved in the pathological activation of cell death pathways in AD. We discuss these findings and their role in the search for new disease treatments.

Keywords
Alzheimer's disease · Amyloid-beta 42 · Natural products · Lunasin · Natural products · Animal model · Neuroprotective · Anti-inflammation · Antioxidant · *Drosophila* · Cell death · Neurodegeneration

Introduction

Alzheimer's disease (AD) is a fatal neurodegenerative disorder that predominantly affects people aged over 65 years, an age group that is expected to increase substantially in the future (Ortman et al. 2014). AD is prevalent, affecting around 10% of people in the USA aged above 65 years, and is expected to almost triple by the year 2060 (Hebert et al. 2013; Matthews et al. 2018). AD presents a major threat as people may live with AD for years – typically 4–8 years after diagnosis, although some people may live up to 20 years. The pathological changes associated with AD may begin decades before symptoms are seen (Alzheimer's Association 2018). AD is marked by severity and persistence in cognitive decline that substantially affects a person's ability to perform daily activities, which begins as mild motor issues and progresses into substantial cognitive errors, such as problems with word finding, or inability to recognize family members, and later, people often become completely dependent on their caretakers. AD drastically affects the quality of life of those suffering from it and creates a phenomenal emotional and financial burden on their friends and family.

In 1906, Dr. Alois Alzheimer first reported shrinkage of the brain in the autopsy of the patient who suffered from dementia (Fig. 1). Various milestones in understanding the cause of AD and its treatment regimen are listed in Fig. 1. AD, a neurodegenerative disorder, is caused by multiple mechanisms, which are likely a combination of genetic and environmental factors. Although a substantial amount is known about the molecular mechanisms associated with AD, there is no cure to date. Furthermore, clinical trials have often shown unsatisfactory results. For this reason, there is a need for disease models that allow us to find new treatment targets quickly and efficiently. The purpose of this chapter is to outline the current state of AD disease and describe the use of *Drosophila melanogaster,* an animal model, in understanding the cause of AD and generating new treatments for AD. Here we

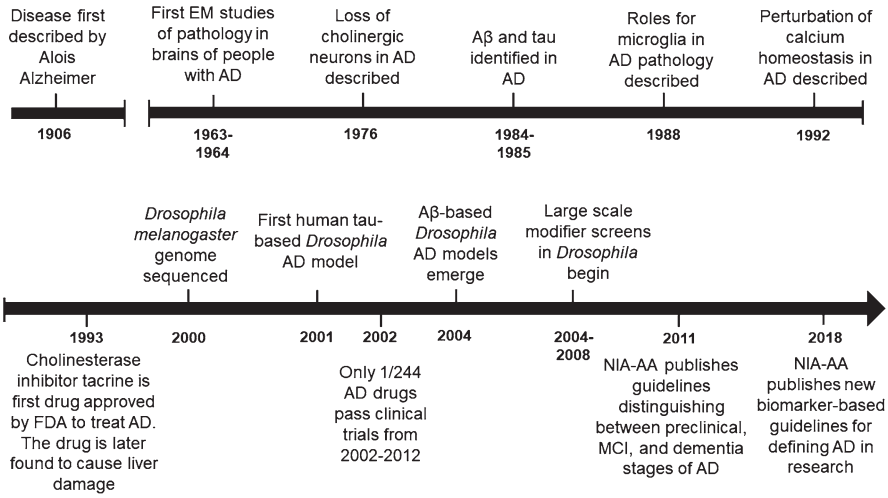

Fig. 1 Abbreviated timeline of AD research and its intersection with *Drosophila* research. *Drosophila* research has evolved rapidly, facilitating the use of large-scale modifier screens to search for new AD treatment targets. *NIA-AA* National Institute on Aging and Alzheimer's Association, *MCI* mild cognitive impairment

provide an overview of recent insights into the role that cell death signaling plays in disease pathology.

Pathology of AD

Initial investigations into AD noted anatomical changes indicative of widespread neurodegeneration, such as decrease in the size of the cerebral cortex and concomitant enlargement of the ventricles (McKhann et al. 1984). Certain areas of the brain are preferentially affected by AD, and it is not known how the disease spreads through the brain (Fig. 2). However, protein misfolding and aggregation appear to be a major part of disease progression. Two key characteristics of the disease are amyloid beta plaques (Aβ42 plaques, also called senile plaques) and neurofibrillary tangles (NFTs) (Figs. 2 and 3). There are numerous other pathological changes associated with AD, including widespread inflammation, reactive gliosis, perturbation of calcium homeostasis, and mitochondrial dysfunction (Cline et al. 2018; Hansen et al. 2018; Shirwany et al. 2007). The causal relationships among these elements of the disease are not fully understood and may vary among brain regions and among individuals. The result, however, is a disease state of widespread cell death in the brain. We will focus on Aβ and neurofibrillary tangles as they are commonly associated with AD and used to model the disease in animal research.

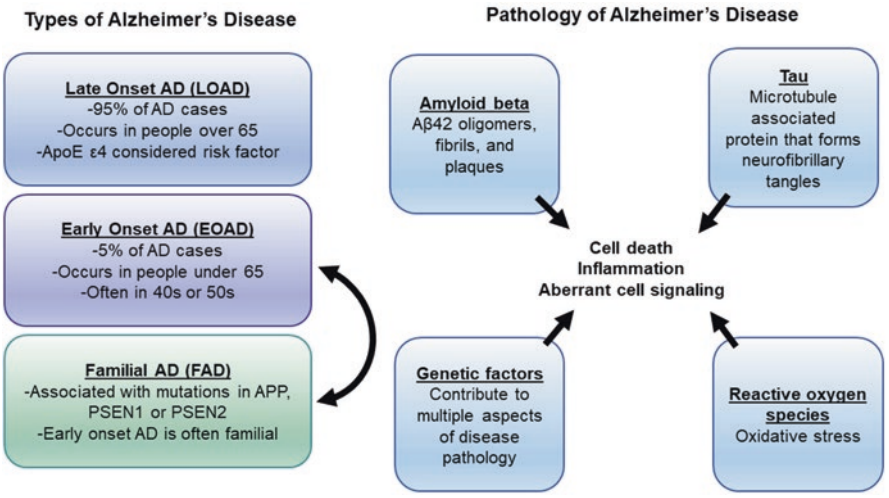

Fig. 2 Overview of the types of AD and pathology involved. AD can be categorized as late onset, early onset, or familial. Early-onset AD is frequently familial and may be called EOFAD. Many factors involved in AD pathology have been identified, and the best understood aspects of pathology are Aβ42, tau, and reactive oxygen species. Genetic factors contribute to AD pathology in multiple ways, with currently the most understood of the genetic factors is related to Aβ42 production

Tau and Neurofibrillary Tangles

Improper regulation of the tau protein, a microtubule-associated protein (MAP), is one of the components of AD. This aspect is shared among several neurodegenerative disorders like Parkinson's disease and Huntington's disease (Chang et al. 2018; Gratuze et al. 2016). AD is associated with the formation of intracellular NFTs comprising the hyperphosphorylated tau protein (Figs. 2 and 3) (Grundke-Iqbal et al. 1986; Kosik et al. 1986; Lee et al. 1991; Wood et al. 1986). Tau plays a vital role in a normal, healthy brain, supporting axonal transport by stabilizing microtubules. It is commonly observed in neurons and also in astrocytes and oligodendrocytes (Migheli et al. 1988; Müller et al. 1997; Papasozomenos and Binder 1987).

Amyloid Beta 42 (Aβ42)

Aβ42 plaques and aggregates are found in the brains of AD patients and are accepted as sources of disease pathology (Glenner and Wong 1984; Hardy and Selkoe 2002; Jack et al. 2018; Klunk et al. 2003; Masters et al. 1985; Villain et al. 2012). Aβ42 is a cleavage product of amyloid precursor protein (APP). APP can be cleaved by α-secretase or β-secretase. The α-secretase cleaves APP in the middle of the Aβ sequence and produces peptides that are not pathogenic. However, cleavage of APP by β-secretase and γ-secretase produces Aβ42 (Figs. 2 and 3). Oligomers of Aβ vary

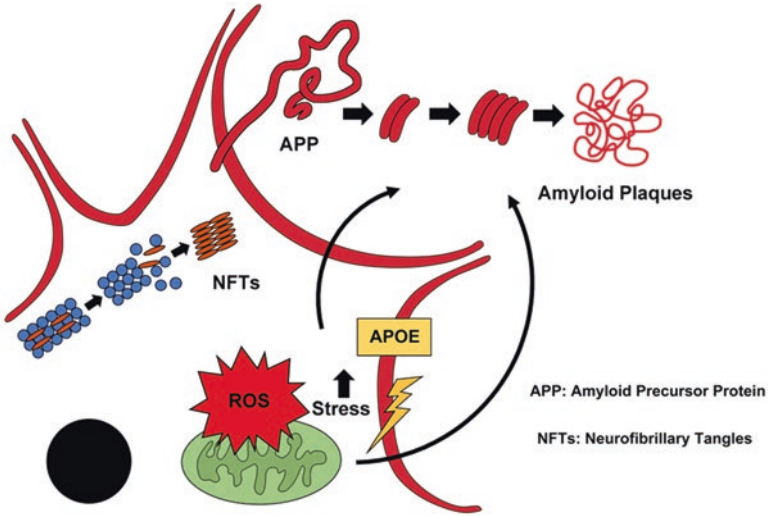

Fig. 3 Overview of the various mechanisms responsible for AD. A transmembrane protein, amyloid precursor protein (APP), is cleaved into Aβ42, which forms oligomers and eventually aggregates into amyloid plaques. Normally, microtubules (blue circles) are associated with tau (red), a microtubule-associated protein (MAP). In AD, tau is hyperphosphorylated, which aggregates and deposits in the AD brain as neurofibrillary tangles (NFTs). The APOE ε4 allele, a major cholesterol carrier, affects amyloid-beta aggregation and clearance that may exacerbate other disease processes. Lastly, oxidative stress and mitochondrial dysfunction result in the generation of reactive oxygen species (ROS) that trigger inflammation and AD

in size and are described by the length of the polypeptide (e.g., Aβ35, Aβ40, Aβ42, or Aβ51). The most common forms are Aβ40 and Aβ42, of which the Aβ42 form is implicated in AD pathology. Aβ42 is hydrophobic and prone to aggregation. Aβ42 oligomers form insoluble fibers, which are the basis for extracellular senile plaques (Fernandez-Funez et al. 2013; Sarkar et al. 2016; Selkoe and Hardy 2016). Shorter forms do not aggregate and are generally regarded as more benign.

Aβ42 oligomers that exhibit neurotoxicity have been associated with a variety of forms of pathology including oxidative stress, inflammation, axonal transport defects, and cell death (Cline et al. 2018; Selkoe and Hardy 2016). People with the Osaka familial AD mutation have fewer senile plaques but more Aβ oligomers in their cerebrospinal fluid and experience significant cognitive impairment (Cline et al. 2018; Kutoku et al. 2015; Tomiyama et al. 2008). Similarly, a mouse model was designed in which APP produced isoforms that yielded either oligomers but not plaques or both oligomers and plaques. Oligomers alone and oligomers with plaques both showed equivalent levels of pathology (Gandy et al. 2010). A related hypothesis suggests that some of the pathologies of Aβ oligomers are due to their ability to form ion channels in cells. Lack of regulation of calcium influx into cells could trigger apoptosis and lead to widespread cell death (Casas-Tinto et al. 2011). Aberrant calcium channels formed by Aβ42 could also explain the depolarization of synaptic membranes seen in some AD models (Abramov et al. 2004; Mirzabekov et al. 1994).

Genetic Risk Factors

While most cases of AD appear sporadically in older populations, there are several known genetic risk factors. People with close relatives who have AD are at a higher risk for the disease (Loy et al. 2014). Early-onset AD occurs in people aged below 65 years. Late-onset Alzheimer's disease (LOAD), occurring in people aged above 65 years, accounts for around 95% of AD cases (Fig. 2) (Isik 2010). Early-onset familial AD (EOFAD) occurs in people aged under 65 years and often involves a mutation in APP, or presenilin 1 or 2, which form part of the γ-secretase complex that cleaves APP (Lleó et al. 2002; Wu et al. 2012). APP is located on chromosome 21 in humans; the same chromosome triplicated in Down syndrome. People with Down syndrome appear to accumulate Aβ at a higher rate, and AD is much more common in this group (Glenner and Wong 1984; Hartley et al. 2017).

Genome-wide association studies (GWAS) have identified a host of other factors that may be related to the development of AD. Autophagy defects may predispose people to AD through failure to clear Aβ, allowing it to aggregate (O'Keefe and Denton 2018). One isoform of the lipid-binding protein apolipoprotein E (ApoE) is considered a risk factor for late-onset AD: ApoE ε4 (Bagyinszky et al. 2014). ApoE ε2 is considered protective and ApoE ε3 neutral. ApoE isoforms can be informative for grouping people in clinical trials, as the efficacy of certain therapies may depend on an individual's ApoE isoform. In order to validate the role of these causative agents in AD and to understand the molecular mechanism, in vivo animal model systems are needed.

AD Animal Models

Numerous animal models of AD exist, which typically focus on recapitulating the disease by manipulating APP, Aβ42, tau, or presenilin 1 (Abramov et al. 2004; Fernandez-Funez et al. 2013; Jankowsky and Zheng 2017; Pandey and Nichols 2011; Sarkar et al. 2016). Some models use an organism's homologs of disease genes, while others use the transgenic expression of human genes (Table 1). Rodent models have many benefits for studying human neurodegenerative diseases. The brains of mice and rats are similar in structure to those of humans, and rodents exhibit a range of complex behaviors for which well-established tests exist. Mouse models usually involve transgenic mutation of APP, presenilin 1, or tau. One of the most commonly used mutants is the transgenic line Tg2576, which uses overexpression of a mutant APP. These mice show Aβ42 plaques and develop cognitive defects. Other common models include TgCRND8 (another APP mutant line), APPswe/PS1ΔE9 (a double mutant of APP K670N, M671L, and PSEN1), and 3xTgAD (a triple mutant of APP, PSEN1, and tau) (Jankowsky and Zheng 2017). Rodent models remain invaluable as mammalian systems for validation of research findings prior to clinical trials. However, in AD research, many treatments that have shown promise in rodent models have failed at the clinical trial level (Goldman et al. 2018). Costs, time constraints, and the intensity of personnel training required for the use of rodents make them less than ideal for high-throughput screens.

Table 1 Overview of notable and commonly used AD models in *Drosophila* and other organisms

Organism	**Modeling strategy**
Caenorhabditis elegans	Human Aβ expression in muscle (Link 1995)
	Human WT and FAD PSEN1 and PSEN2 mutants (Levitan et al. 1996)
	Overexpression of the APP homolog APL-1 (Hornsten et al. 2007)
	Expression of Aβ42 in glutamatergic neurons (Treusch et al. 2011)
Danio rerio	Manipulation of zebrafish homologs *psen1* (Nornes et al. 2003) and *psen2* (Nornes et al. 2008)
	Translation blocking of APP homologs *appa* and *appb* (Joshi et al. 2009) Aβ-level reduction (Luna et al. 2013)
Mus musculus	Transgenic lines Tg2576 (APPswe) (Hsiao et al. 1996)
	TgCRND8 (APPswe/ind) (Chishti et al. 2001)
	APPswe/PS1ΔE9 (Jankowsky et al. 2004)
	3XTg-AD (APPswe, PSEN1 M146V, and tau P301L) (Oddo et al. 2003)
Rattus norvegicus	Transgenic strains with FAD-associated mutations: UKUR28 (APPswe and APP V717F), UKUR19 (PSEN1 M146L), and UKUR25 (APP/PSEN1 double mutants) (Echeverria et al. 2004)
	TgF344-AD transgenic strains with the FAD-associated mutations: APPswe and PS1ΔE9 (Cohen et al. 2013)
***Drosophila melanogaster* Targets**	**Transgenic expression strategy**
dTau	dTau overexpression (Mershin et al. 2004)
Human tau	WT and mutant R406W and V337M tau (Wittmann et al. 2001)
	Phospho-mimetic Tau^{E14} (Khurana et al. 2006)
	Non-phosphorylatable Tau^{S2A} and Tau^{S11A} (Chatterjee et al. 2009)
Aβ	Expression of Aβ40 and Aβ42 (Finelli et al. 2004)
	Expression of WT and Arctic mutant E22G Aβ42 (Crowther et al. 2005)
APP	WT APP, APPswe, and APP with truncated C-terminal (Fossgreen et al. 1998)
APPL	APPL overexpression (Carmine-Simmen et al. 2009; Torroja et al. 1999)
dBACE	dBACE expression (Carmine-Simmen et al. 2009)
Human BACE	Human BACE expression (Greeve et al. 2004)
dPsn	dPsn with FAD-associated mutations (N141I, M146V, L235P, and E280A) (Ye and Fortini 1999)

Citations refer to the first publication of the models

AD models also exist for zebrafish, *Danio rerio,* and roundworm, *Caenorhabditis elegans* (Table 1) (Alexander et al. 2014; Newman et al. 2014). Zebrafish have the translational benefits of being vertebrates but are somewhat costly to care for and have a relatively long 90-day life cycle. *Caenorhabditis elegans* remains extremely useful for basic science approaches including studying molecular mechanisms of AD; however, they lack centralized brains and are relatively limited in terms of

behavioral studies. While these systems have great potential for modeling AD, *Drosophila melanogaster,* a highly versatile genetically tractable model, holds a lot of promise to understand molecular-genetic underpinnings of AD and other neurodegenerative disorders (Table 1). Fruit flies provide a convenient set of tools to genetically dissect the pathways involved in AD and provide a good compromise between similarity to humans and ease of use. *Drosophila* also has the substantial advantage of both gene expression tools that can be induced at specific points in development and a short life cycle. These features render *Drosophila* useful for finding both treatments that prevent AD-related pathology and those that may reverse pathological changes that have already taken place.

Utility of *Drosophila* as a Model System

Drosophila has many advantages for studying neurodegenerative disorders including AD (Bonini and Fortini 2003; McGurk et al. 2015; Sarkar et al. 2016; Singh and Irvine 2012). Lower redundancy in the genome makes it easier to observe phenotypes in lower organisms than in higher organisms. The flies exhibit substantial homology with humans, including homologs for around 70% of the genes commonly associated with human diseases (Bier 2005; Reiter et al. 2001; Sarkar et al. 2016; Singh and Irvine 2012). Furthermore, the synaptic vesicle release machinery is well-conserved between flies and humans, rendering them useful for both basic science studies into neuronal activity and disease modeling. The barrier for the use of *Drosophila* in research is low. Fly stocks can be maintained cheaply and do not require much space. The ease of use of *Drosophila* in terms of training new personnel is also worth noting. *Drosophila* is highly accessible for use in labs at primarily undergraduate institutions as well as at other research institutions. Basic fly husbandry requires training to identify sex and visible markers. For screens based on visible phenotypes, a considerable amount of work can be accomplished with relatively little training time. Eye phenotypes are often readily apparent, and screens may be used to identify modifiers.

Flies go through multiple distinct stages of development. After hatching from their eggs, the larvae quickly increase in size through the first, second, and third instar stages. The larva houses the blueprint of adult appendages referred to as the imaginal discs (Cohen 1993; Held 2002; Singh et al. 2005, 2012; Tare et al. 2013). The larva metamorphoses into the pupa, and the adult fly eventually emerges from the pupal case. These stages provide multiple options for study. Larval preparations are highly accessible to gene expression, protein localization by immunohistochemistry, protein-protein interactions, and electrophysiological recording. Behavioral and locomotor assays can be performed on larvae or adults.

Adult flies may live around 90 days. Their short life cycles also are an asset in studying age-related neurodegeneration in diseases such as AD (He and Jasper 2014; Iliadi et al. 2012; Sun et al. 2013). Flies exhibit more susceptibility to neurological problems with aging (Reynolds 2018). In this way, it is possible to screen for new treatments at different points in the disease progression and study how natural aging may interact with disease pathology.

The popularity of *Drosophila* has led to the development of a vast array of genetic tools that can be obtained through stock centers. The Gal4-UAS system is a staple of fly genetics. This system makes use of factors originally found in yeast and can be used to express genes of interest in a specified tissue. The upstream activation sequence is fused to a protein of interest, while the Gal4 sequence is fused to a tissue-specific promoter. When the flies containing the UAS sequence are crossed to those with the Gal4 sequence, the Gal4 protein is produced and binds to the UAS sequence in the tissue of interest, promoting transcription (Brand and Perrimon 1993). Another layer of regulation can be introduced by Gal80, a repressor of the Gal4-UAS system (or $Gal80^{TS}$, its temperature-sensitive version). Gal80 binds to Gal4 and prevents transcription of the UAS-linked gene. When $Gal80^{TS}$ is expressed, it prevents transcription of genes at temperatures like 18 °C, whereas at a temperature of 29 °C or above, $Gal80^{TS}$ is inactivated and the gene of interest is now transcribed. This system can be used to temporally regulate the expression of a specified gene (McGuire et al. 2003). If temperature sensitivity is a concern, there is a version of the Gal4-UAS system that can be induced by the presence of the drug mifepristone (RU-486). In this version, transcription of the gene of interest will be active only when the drug is present to bind to the hormone receptor. The drug is typically delivered via the fly food (McGuire et al. 2004).

Generating custom fly stocks is not trivial, but it is a relatively fast process compared to the options available in other systems. Transgenics is well established in flies. Transgenic fly lines may be generated in which a human gene, under UAS control, is inserted into the genome. Other possibilities include the use of the CRISPR/Cas9 system to edit the genome with more specificity. Point mutations can be introduced into *Drosophila* homologs in this way (Bassett et al. 2013). Thus, the fly has been proved to be highly versatile and tractable to model human disease.

Modeling AD in *Drosophila*

Modeling AD in *Drosophila* typically involves the expression of disease-related proteins in certain tissues. Table 1 provides an overview of approaches often used to study AD. Common tissues for expression of disease proteins include the developing retina (GMR-Gal4, Glass Multiple Repeat, Table 2) (Moses and Rubin 1991; Tare et al. 2011), the mushroom bodies (OK107-Gal4, Table 2) (Connolly et al. 1996), or in all neurons ($elav^{C155}$-Gal4, embryonic lethal abnormal vision) (Lin and Goodman 1994). Table 2 summarizes drivers commonly used in studying AD in *Drosophila.* The mushroom bodies are associated with learning and memory in flies, making the expression in this area useful for studies on olfactory learning. The pan-neuronal expression can be used to study the global effects of disease proteins on the fly nervous system, while expression in the developing retina typically results in a rough eye phenotype that can be used for screening. Flies possess many of the same components involved in AD pathology in humans, and some studies overexpress homologs of AD-associated genes. Other studies express the human versions of AD-related proteins such as Aβ42 or tau (Fernandez-Funez et al. 2013; Pandey and Nichols 2011; Sarkar et al. 2016).

Table 2 Summary of the driver lines used in modeling AD in *Drosophila*

Gal4 driver	Expression pattern	Source
GMR-Gal4	Developing retina	Moses and Rubin (1991)
elav^{C155}-Gal4	Pan-neuronal	Lin and Goodman (1994)
Appl-Gal4	Pan-neuronal	Torroja et al. (1999)
OK107-Gal4	Mushroom bodies	Connolly et al. (1996)
repo-Gal4	Glia	Sepp et al. (2001)
eyeless-Gal4	Eye	Hazelett et al. (1998)
A307-Gal4	Giant fiber system	Phelan et al. (1996)

Flies have a tau homolog, which is required for viability and the normal development of the eye and nervous system (Tan and Azzam 2017). Tau knockdown causes lethality, with 3% of escapers eclosing as adults, and its impairment leads to neurodegeneration (Bolkan and Kretzschmar 2014). Gain-of-function of dtau in mushroom bodies results in loss of learning and memory (Table 1) (Mershin et al. 2004). An early study expressed a GFP-tagged bovine tau in *Drosophila* sensory neurons and saw several defects including developmental loss of axons and a decrease in arborization (Williams et al. 2000). Expression of wild-type tau and a mutant form of tau associated with familial dementia led to neurodegeneration, lethality, and accumulation of the protein. Animals with mutant tau showed stronger phenotypes, although, interestingly, NFTs were not observed in this model (Wittmann et al. 2001). Tau overexpression appears to trigger neurodegeneration in part through the accumulation of filamentous actin (Fulga et al. 2007).

Flies have homologs of several of the genes required to process Aβ42 including a gene similar to APP called APP-like (APPL) (Fossgreen et al. 1998; Luo et al. 1992; Wasco et al. 1992). Flies have a presenilin homolog (dPsn) (Table 1) (Struhl and Greenwald 1999; Ye and Fortini 1999; Ye et al. 1999), as well as an α-secretase called Kuzbanian (kuz) (Rooke et al. 1996). Kuz is able to cleave APPL (Carmine-Simmen et al. 2009). Flies also have an enzyme with β-secretase activity (dBACE, β-site APP-cleaving enzyme) that can also cleave APPL and produce neurotoxic amyloid (Table 1) (Carmine-Simmen et al. 2009; Greeve et al. 2004). APPL, however, lacks the specific Aβ42 domain found in humans (Luo et al. 1992). Several early studies looked at the overexpression of these proteins in flies. One study overexpressed *Drosophila* APPL along with bovine tau and saw defects in axonal transport (Torroja et al. 1999). Another study overexpressed human APP in *Drosophila* imaginal discs, which triggered a blistered wing phenotype (Yagi et al. 2000). Expressing human BACE and human APP in the developing retina in flies led to amyloid plaque formation and neurodegeneration. Addition of *Drosophila* presenilin with a mutation associated with familial AD worsened the neurodegeneration (Greeve et al. 2004). Similarly, other early studies compared overexpression of wild-type Aβ42 with the Aβ42 Arctic mutant, which featured a mutation associated with another familial form of AD. Use of the Arctic mutant triggered severe phenotypes as compared to the wild-type Aβ42 expression (Crowther et al. 2005). All these studies in flies established *Drosophila* as a suitable model to study AD pathology and progression.

The effects of differentially expressing Aβ40 and Aβ42 have also been examined. Pan-neuronal expression of Aβ42 led to neurodegeneration in which amyloid deposits could be observed, as well as increased mortality and age-dependent defects in olfactory learning. By contrast, pan-neuronal expression of Aβ40 resulted only in age-dependent learning defects (Iijima et al. 2004). Further research into the differences between short and long Aβ peptides supports the conclusion that Aβ42 is the primary source of AD pathology. Peptides with 36–40 amino acids in length do not cause defects in the eye structure and do not form plaques. When expressed in addition to Aβ42, these shorter peptides have a mild protective effect and can partially rescue the eye morphology and motor deficits (Moore et al. 2018).

Drosophila Eye Model

The eye is an excellent model for neurodegeneration studies, as it is not required for viability and mutations often yield visible phenotypes (Cutler et al. 2015; Iijima-Ando and Iijima 2010; Lenz et al. 2013; Moran et al. 2013; Steffensmeier et al. 2013; Tare et al. 2011). The eye-antennal imaginal disc provides the tissue for the compound eye of the adult fly. The signaling pathways involved in *Drosophila* eye development are well-characterized. The adult eye comprises 750–800 ommatidia, each with 8 photoreceptors (Kumar 2011; Ready et al. 1976; Singh et al. 2012; Tare et al. 2013). One major advantage of the *Drosophila* eye model is that the eye is not required for viability (Sarkar et al. 2016). Adult flies can survive with severely malformed eyes or no eyes at all. This system affords researchers the opportunity to study genes that may be lethal if expressed more widely throughout the animal – and to study those genes specifically in a neuronal model. Interestingly, AD can damage the neurons that make up the retina in humans, leading to visual disturbances. Recently, new detection strategies have been developed, which are not as expensive as commonly used PET scans. These eye scan techniques detect Aβ42 deposits in the retina using noninvasive retinal scans and may allow early detection of AD (Colligris et al. 2018).

Human Aβ42 can be expressed in the eye using the Gal4/UAS system. One of the common approaches is to use the driver GMR-Gal4, which drives expression in differentiating retinal neurons subsequent to the activation of retinal determination genes (Fig. 4) (Moses and Rubin 1991; Tare et al. 2011). Expression of a UAS-Aβ42 transgene using the GMR-Gal4 driver results in animals with highly reduced and glassy eyes due to neurodegenerative defects in their ommatidia (Fig. 4). These animals also show extracellular Aβ42 plaques analogous to what is seen in the brains of AD patients (Casas-Tinto et al. 2011; Moran et al. 2013; Steffensmeier et al. 2013; Tare et al. 2011). Under certain conditions (e.g., raising animals at 29 °C), this effect is 100% penetrant. Furthermore, this neurodegenerative phenotype is progressive in nature (Tare et al. 2011). There are several different Aβ42 overexpression lines available. Commonly used lines include UAS-Aβ42^{2X}, UAS-Aβ42^{11C39}, UAS-Aβ42$^{H29.3}$, and UAS-Aβ42^{BL33770}. When expressed in the developing retina, these lines vary in terms of cell death, lethality, and severity of their eye

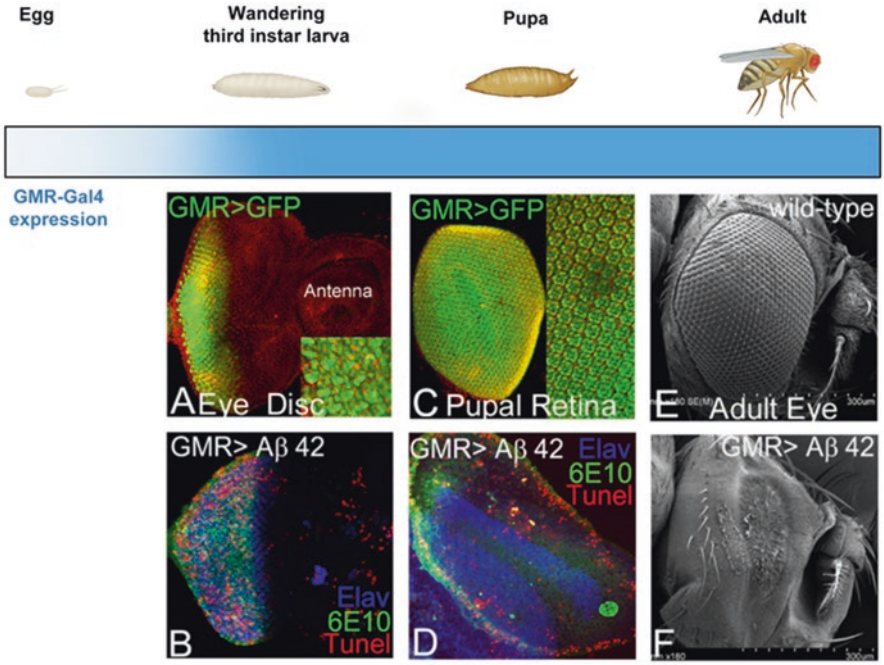

Fig. 4 Targeted misexpression of human Aβ42 in *Drosophila* eye triggers neurodegeneration as seen in AD. Using GMR-Gal4>Aβ42 to model AD in *Drosophila*. GMR-Gal4 expression turns on during the third instar larval stage. (**a**) Using GMR-Gal4 to drive UAS-GFP (GMR>GFP) triggers expression in the differentiating retinal cells of the larval eye disc and (**c**) in the entire pupal retina. (**b**) GMR-Gal4 drives expression of Aβ42 in the differentiating neurons of the eye disc, triggering Aβ42 accumulation (marked by 6E10 antibody, green). Elav (blue) marks all neurons and TUNEL (red) marks cell death. (**d**) 72 h pupal retina; the same staining as in (**b**). Cell death can be observed in the pupal retina 28 h after pupal formation. (**e**) Eye of adult wild-type fly. (**f**) GMR>Aβ42 flies show pronounced neurodegeneration compared to wild-type flies

phenotypes. These differences were compared in a recent study (Jeon et al. 2017). Aβ42 expression in flies consistently leads to a neurodegenerative profile consistent with AD and, furthermore, often results in phenotypes that can be easily screened under the stereomicroscope. The *Drosophila* model also possesses an excellent capacity for drug discovery through high-throughput screening (Fernandez-Funez et al. 2013; Pandey and Nichols 2011) as well as for genome-wide genetic screens (Moran et al. 2013; Sarkar et al. 2016).

Suitability of *Drosophila* Model for Screens

Drosophila has historically been associated with high-throughput, genome-wide screens, and this use remains highly relevant to AD research (Bellen et al. 2010; Lenz et al. 2013). Screens provide the first round of insight into new treatments. Standard screens fall into the categories of drug and genetic screens.

Drug Screens

Drosophila provides an excellent system for testing and screening for putative drug targets for AD in high-throughput screens. One study combined high-throughput screening in cell culture with validation in a *Drosophila* pan-neuronal Aβ42 model. After screening 65,000 small molecules, one called D737 was capable of mitigating Aβ42 toxicity and improving fly lifespan (McKoy et al. 2012). The *Drosophila* eye model for AD can also be used to screen for putative drug targets (Singh, unpublished). The rationale is to screen for inhibitors of Aβ42 toxicity. The drugs or chemical inhibitors can be mixed in DMSO in cornmeal agar food (Gladstone and Su 2011). It has been determined that larvae can tolerate 0.10% or lower of DMSO in cornmeal agar food. Therefore, we can use the drugs or chemical inhibitors at a 1000-fold dilution that is 1 μM (for those available as 1 mM stock) or 1 and 10 μM (for those available as 10 mM stock). The screen is based on the fact that if a chemical inhibitor can block Aβ42 toxicity, then third instar larvae, where high levels of Aβ42 have been expressed in differentiating retinal neurons when fed these chemical inhibitors in food, will restore the highly neurodegenerative phenotype (Figs. 4f and 5) to near wild-type eye (Figs. 4e and 5). The *Drosophila* eye phenotype can be

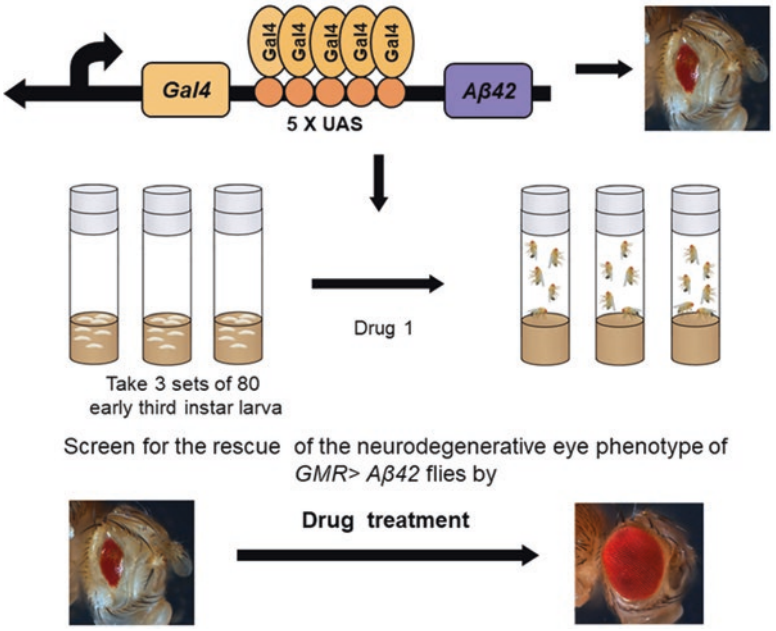

Fig. 5 Strategy for drug screen to identify modifiers of gain of function of Aβ42 (GMR-Gal4>Aβ42) in the *Drosophila* eye. First, 80 early third instar GMR-Gal4>Aβ42 larvae are collected in food vials. Larvae are subjected to drug treatment and observed as adults for rescue of the Aß42 neurodegenerative eye phenotype. Each sample is tested in triplicate to prevent variation in handling (Singh, Unpublished)

scored easily. An outline of the drug screen is provided in Fig. 5. A pilot screen using known chemical inhibitors of c-Jun N-terminal kinase (JNK) signaling, which is known to trigger cell death in Aβ42-mediated neurotoxicity, was tested. These inhibitors can block Aβ42-mediated neurotoxicity. Thus, the *Drosophila* eye model can be used to screen the chemical libraries for potential therapeutic targets for AD.

Genome-Wide Genetic Screens

The genetic screens can be further classified into forward or reverse genetics. Since a considerable amount is known about the individual biochemical facets of AD, simpler model systems provide the first step in a pipeline to develop new treatments. To date, there have been several large-scale screens undertaken to uncover modifiers of the Aβ42-induced pathology. The outcome of these screens has revealed a considerable amount of the mechanisms that lead to neurodegeneration in these flies. In one such screen, around 2000 EP transposon lines were examined, resulting in the identification of 23 modifiers. These modifiers ranged in function and included genes affecting lysosomal transport, secretory pathways, signal transduction, and chromatin regulation (Cao et al. 2008; Finelli et al. 2004). Another group performed a large-scale screen of a collection of 3000 Gene Search insertion lines for genes that increased the longevity of flies pan-neuronally expressing the Aβ42 Arctic mutation. They found that oxidative stress contributes to Aβ42 toxicity, which can be ameliorated through the iron-binding capabilities of the protein ferritin (Rival et al. 2009). Later studies from the same group showed that expression of puromycin-sensitive aminopeptidase was also able to improve lifespan and aided in Aβ42 clearance (Kruppa et al. 2013).

One of the screens examined a set of second and third chromosome deficiency lines in the GMR-Gal4>Aβ42 (where high levels of human Aβ42 are expressed in retinal neurons) background and found 14 suppressors and 9 enhancers. One of the genes uncovered was *Toll*, which has a canonical role in NFκB signaling in inflammation and immunity, a pathway conserved between flies and humans (Tan et al. 2008). Interestingly, *Toll* also was uncovered independently in the previous screen (Cao et al. 2008). Loss of function of *Toll* was found to suppress neurodegeneration, while the gain of function enhanced the phenotype (Tan et al. 2008). The deficiency lines uncovering the third chromosome were used in a screen for modifiers of locomotor defects induced by expressing the Aβ42 Arctic mutation in the giant fiber system (Liu et al. 2015a). Climbing defects triggered by pan-neuronal expression of Aβ42 were also examined in a modifier screen using deficiency lines specifically examining aged flies (Belfiori-Carrasco et al. 2017). A series of reports have described the results from a large-scale screen looking for modifiers of the GMR-Gal4>Aβ42 eye phenotype and led to the identification of members of evolutionarily conserved signaling pathways. These results suggested how the activation of signaling cascades may lead to cell death in AD (Moran et al. 2013; Tare et al. 2011). The rationale of the screen was to overexpress one gene at a time in the GMR-Gal4>Aβ42 background and assay its effect on the neurodegenerative

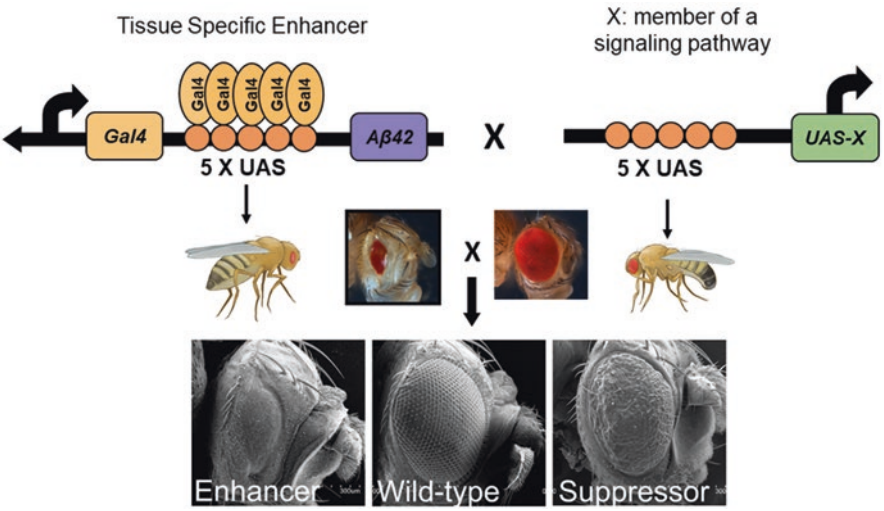

Fig. 6 Strategy for forward genetic screen to identify genetic modifiers of Aβ42 (GMR-Gal4>Aβ42) gain-of-function in *Drosophila* eye. Flies expressing human Aβ42 under the control of the GMR-Gal4 driver (small, rough eyes) are crossed to flies in which genes of interest (X) are expressed under UAS control (normal eyes). Eye phenotypes are then observed in the progeny to determine whether a given gene has acted as an enhancer or suppressor of the Aβ42 eye phenotype

phenotype (Fig. 6). The genetic modifiers were classified into enhancers or suppressors based on their capability to enhance or suppress the neurodegenerative phenotype of GMR-Gal4>Aβ42 (Fig. 6). This screen resulted in the identification of members of evolutionarily conserved signaling pathways. The results from this screen suggested that accumulation of Aβ42 plaques can trigger aberrant signaling, which results in neurodegeneration.

Aberrant Activation of Cell Death Pathways

Expression of Aβ42 in the retina triggers neurodegeneration that can be observed at multiple stages of development. Eye-antennal imaginal discs show organizational defects, such as fused or disorganized ommatidia. Large vacuoles in the retinal tissue can be observed later in development (Fig. 4). The TUNEL staining showed that these flies that express high levels of human Aβ42 undergo substantially more cell death. This neurodegeneration is mediated at least in part by activation of c-Jun N-terminal kinase (JNK) signaling (Tare et al. 2011).

JNK activates c-Jun, an immediate early gene, by phosphorylation. c-Jun binds to c-Fos and forms a heterodimer (Karin et al. 1997). c-Jun phosphorylation can be used as a measure of JNK activity. Levels of *puckered* (*puc*), a gene downstream of JNK, can similarly be used to infer JNK activity. Aβ42 flies show increased levels

of both *puc* and phosphorylated Jun. *Puc* also acts as an inhibitor of JNK, and expression of *puc* in Aβ42 flies was able to rescue the neurodegeneration (Martin-Blanco et al. 1998; Tare et al. 2011). Similarly, expression of a dominant negative form of the Jun kinase Basket (Bsk), bsk^{DN}, was also able to restore a normal eye phenotype. Overall, several lines of evidence support a role for JNK signaling in mediating the neurodegeneration seen in Aβ42 flies (Tare et al. 2011).

Similarly, expression of Aβ42 in neurosecretory and epithelial cells was found to trigger caspase activation through Wingless (Wg) signaling (Arnés et al. 2017). Another recent study highlighted roles for glia in clearing Aβ from the extracellular space. Draper is a glial engulfment receptor. Mutations in *draper* further impair Aβ42 flies. This study showed evidence for JNK signaling activation downstream of Draper (Ray et al. 2017).

Chaperone proteins play important roles in protecting against apoptotic cell death by helping refold or otherwise sequester misfolded proteins (Martín-Peña et al. 2018). The chaperone heat shock protein 70 (Hsp70) has been shown to inhibit the activation of JNK, preventing downstream cell death (Jäättelä et al. 1998; Mosser et al. 1997). Hsp70 can bind to Aβ42 and prevent it from forming aggregates. An alternative localization sequence was created to target Hsp70 to the extracellular space where Aβ aggregates form. Expression of this form of the protein in the mushroom body had a number of neuroprotective effects including rescuing lethality and motor defects, decreasing cell death, and restoring normal structure to the mushroom body (Fernandez-Funez et al. 2016). A further study found that this form of Hsp70 was able to rescue the learning deficits seen in Aβ42 expressing flies (Martín-Peña et al. 2018).

Screens for modifiers of the Aβ42 phenotype also found that the homeotic gene *teashirt* (*tsh*) and its paralog *tiptop* (*tio*) act as suppressors of cell death. *Tsh* expression in the retinal neurons restores the Aβ42 phenotype to a wild-type eye phenotype and rescues axonal targeting from the retina to the brain. These functions appear to be genetically separable from eye development (Moran et al. 2013). The CREB-binding protein (CBP) was also found to have a neuroprotective role. The high level of expression of CBP, a histone acetylase, in the retina in Aβ42 models was found to rescue neurodegeneration and axonal targeting defects seen in these flies. The domains were genetically dissected, and it was found that the Bromo, HAT, and polyQ domains were required for its neuroprotective effects (Cutler et al. 2015).

Other studies have found enhancers of the neurodegenerative phenotype. *Crumbs* (*crb*) is the apical-basal cell polarity gene and was found to be upregulated in the Aβ42 background. Expression of a full-length *crb* construct in an Aβ42 background led to worsened neurodegeneration as well as increase cell death and axonal targeting deficits (Steffensmeier et al. 2013). Inhibition of calcineurin has also been shown to worsen the Aβ42 phenotype. *Sarah* (Sra) is a calcineurin inhibitor seen to be upregulated in Aβ42 flies. Overexpression of *sra* led to an increase in cell death and worsened the eye morphology phenotype. Treatment with calcineurin-inhibiting compounds or knockdown of calcineurin itself had similar effects (Lee et al. 2016). Thus, identification of members of several signaling pathways and genes responsible for various functions in the cells justifies the existing hypothesis of the presence of multiple factors responsible for AD.

Current Treatments

Even with the wealth of research on AD, few of the FDA-approved treatments available provide more than modest relief. Acetylcholinesterase inhibitors such as donepezil, galantamine, or rivastigmine are commonly prescribed. These drugs help improve cognition by inhibiting the breakdown of acetylcholine. Similarly, the NMDA channel blocker memantine is prescribed, which binds to NMDA receptors to decrease the flow of calcium into the cell. These drugs have also been tested in AD animal models: memantine was tested in an olfactory memory assay in flies pan-neuronally expressing Aβ42 and was found to improve memory, providing additional validation that drug therapies tested in flies can translate to humans (Wang et al. 2012). These drugs are moderately effective in treating cognitive dysfunction, particularly earlier on in the disease. They do not treat the underlying pathology or slow disease progression.

Proteins required to produce Aβ42 are logical targets for interventions that could potentially treat the disease itself. Unfortunately, drugs that show promise ameliorating disease phenotypes in animal models have an extremely high rate of failure in clinical trials. Treatment with the γ-secretase inhibitor semagacestat was associated with cognitive decline as well as a higher risk of skin cancer (Doody et al. 2013). Another drug, tarenflurbil, was proposed to modulate γ-secretase to make shorter and less toxic forms of Aβ, but showed no benefit in clinical trials (Marder 2010). Several current clinical trials have suggested that certain antibodies like aducanumab can bind to Aβ42 aggregates and thereby decrease the amounts of both soluble and insoluble Aβ42 to mitigate its toxicity and potentially slow the course of the disease (Sevigny et al. 2016). Other antibodies intended to target Aβ42, bapineuzumab and solanezumab, failed in clinical trials (Gold 2017). Other single-chain variable fragment antibodies, which are small molecules designed to pass into the brain targeting Aβ42, were capable of rescuing age-dependent memory defects in flies expressing Aβ42 in the mushroom bodies, the brain structure associated with learning and memory (Martin-Peña et al. 2017).

It is unclear whether the lack of promising results from clinical trials indicates issues stemming from the use of animal models or with the clinical trials themselves. Animal models are often able to deliver the treatment concurrently with the disease-causing agent, such as in transgenic models in which a therapeutic protein is expressed in the organism alongside overexpression of tau or Aβ42. These approaches are extremely useful for screening, but do not necessarily reflect the disease progression in humans. In humans, the treatment often comes long after the onset of the disease, especially given that the actual onset of disease pathology could have been years before symptoms were clinically apparent (King 2018). One possibility is that some trials have used participants whose diseases have already progressed too far for certain treatments to be useful. Another potential issue is that AD pathology may vary greatly among individuals. Current diagnostic tools can identify plaques in the brain using imaging as well as the presence of biomarkers like Aβ42 and phosphorylated tau in the CSF, while genetic testing can identify known risk factors (Ceravolo et al. 2008; Mattsson et al. 2009). While informative,

these factors do not give a full picture of what is causing neurodegeneration at a cellular level. The utility of animal models is that we can test very specific disease states for new treatments. Until we can better understand individual differences in AD pathology in humans, we can use animal models to find new therapies that may eventually be combined to tailor treatment to each person with AD.

Natural Products

There are many foods and spices purported to have therapeutic value. Since these compounds occur in food, we already know them to be tolerated by the body at least in some concentrations. Several active compounds isolated from food products have been tested and shown to have therapeutic value in fly AD models, demonstrating some ability to rescue neurodegeneration. The soy protein Lunasin was also found to have a neuroprotective role in the Aβ42 eye model. Previous research has established that Lunasin has anti-inflammatory properties and some capacity for preventing metastasis in cancer models. Expressing lunasin in the Aβ42 model prevented neurodegeneration of the eye and rescued axonal targeting. Lunasin expression also decreased the lethality seen in Aβ42 flies. As in the previous research, lunasin seems to be blocking cell death through downregulation of JNK signaling, with no effect on Aβ plaque accumulation itself (Sarkar et al. 2018).

Cinnamon and turmeric have been touted as folk remedies for a variety of ailments. Cinnamaldehyde, one of the active compounds in cinnamon, was examined in *Drosophila* AD models. Treatment with cinnamaldehyde improved lifespan in tau overexpression flies, but not in Aβ42 flies (Pham et al. 2018). Compounds extracted from the rhizomes of the turmeric plant (*Curcuma longa*) were tested in flies expressing human BACE-1 and APP. Feeding flies curcuminoid compounds showed the capability of rescuing morphological and locomotor deficits (Wang et al. 2014). Flavonoids, the compounds that give plants their pigmentation, were examined in a computational screen for Aβ42 inhibitors. One flavonoid was found to ameliorate defects caused by expressing Aβ42 in the fly eye, and treatment with the compound improved lifespan and locomotion (Singh et al. 2014). One study examined plants associated with traditional Chinese medicine for neuroprotective roles in AD models (Liu et al. 2015b).

Conclusions

Drosophila melanogaster has a long history of use as a screening tool and remains a highly accessible model organism for studying the molecular mechanisms behind neurodegenerative disease. Evidence has emerged in the last 5–10 years that the neurodegeneration seen in AD is related to the aberrant activation of signaling pathways, culminating in cell death. The *Drosophila* eye model has been invaluable for identifying specific molecular players involved in regulating cell death. Given the variety of processes that play roles in AD pathology as well as the range of

symptoms in the disease, it is likely that therapies will need to be tailored to the individual. Likewise, it has become apparent that many neurodegenerative diseases share similar types of pathology, involving considerable crosstalk among many different signaling pathways. Despite the inherent complexity of AD, recent research has identified many potential targets for new therapies. In the process of finding new treatments for AD, fly research remains an excellent early step in the pipeline.

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Conflict of Interest The authors declare no conflict of interest.

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